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Galleria mellonella: An *in vivo* model for accessing the efficacy of colistin in combination with broad spectrum antibiotics against biofilm forming *Acinetobacter baumannii* infections

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I DECLARATION AND CERTIFICATE

I hereby certify that the work embodied in this thesis is the result of my own investigations except where information obtained by colleagues has been acknowledged. This work has not been accepted in substance for any degree and is not currently submitted in candidature for any other degree.

Alice Rose Gillett

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III ABSTRACT

The emergence of opportunistic nosocomial bacteria *Acinetobacter baumannii*, which causes infections in critically ill patients with compromised immune systems, is one of the most clinically challenging pathogens to treat effectively.

Most nosocomial pathogens grow as monoculture or poly-species biofilms in infections and the biofilm mode of existence for *A. baumannii* may almost certainly contribute to its increased multi-drug resistant (MDR), although resistance can also be attributed to many contributing factors including overuse and misuse of antibiotics in hospitals and the community.

In vitro methods for studying microbial biofilms have developed to include the CDC biofilm reactor, Flow cell devices and MBEC™ but *in vivo* biofilm model development has been very limited. Few simple animal models are available which reflect either the biofilm nature of growth of pathogens or the treatment of infection influenced by biofilm development *in vivo*. Biofilm formation is a critical virulence and defence strategy for bacteria, the present study focuses on working towards establishing a biofilm generated in-vivo model to assess the efficacy of antimicrobials against *A. baumannii* infections.

The present study looks at the effects of broad spectrum antibiotics (BSA) and colistin combinations against lethal planktonic or biofilm *A. baumannii* infections with a pre-clinical insect model, *Galleria mellonella* (Greater wax moth larvae). The work shows colistin to be an important synergist both *in vitro* and in a simple *in vivo* model. This work is the first to describe a model biofilm associated infection in *G. mellonella.*

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1 INTRODUCTION

Emerging as one of the most clinically problematic pathogens due to its resistance to multiple antibiotics, *Acinetobacter baumannii* is increasingly becoming one of the most studied species of the genus *Acinetobacter*. Acinetobacter bacteria are typically opportunistic pathogens, posing as a significant threat to critically ill hospitalised patients with an already compromised immune system. *A. baum*annii infections have also been reported to have been identified in individuals with open wounds, or compromised individuals with medical invasive devices such as urinary catheters. At present 17 species of the genus *Acinetobacter* are recognised, with *A. baumannii* being the most commonly isolated species from human specimens. The organism is an aerobic Gram-negative bacterium, recovered from soil, water, and animals[1-7].

Understanding how prevalent the natural habitats of nosocomial bacteria such as *A. baumannii* enables a greater understanding of the infection characteristics of these bacteria. Most nosocomial pathogens grow as monoculture or poly-species biofilms in infections[8]. The biofilm mode of existence for *A. baumannii* may almost certainly contribute to its increased multi-drug resistant (MDR), although resistance can also be attributed to many contributing factors including overuse and misuse of antibiotics in hospitals and the community[9, 10].

In vitro methods for studying microbial biofilms have developed to include the CDC biofilm reactor, Flow cell devices and MBEC™ (formerly known as Calgary peg) but *in vivo* biofilm model development has been very limited. Few simple animal models are available which reflect either the biofilm nature of growth of pathogens or the treatment of infection influenced by biofilm development *in vivo*[11]. Biofilm formation is a critical virulence and

defence strategy for bacteria and the resent study focuses on establishing a biofilm generated *in-vivo* model to assess the efficacy of antimicrobials against *A. baumannii* infections in a more realistic way than previously described.

Colistin monotherapy has been a last resort in clinical practice, when the use of Beta-lactam, aminoglycoside or quinolone antibiotics are ineffective, to counter serious infection with *A. baumannii* but more recently, resistance has emerged rapidly; groups have also reported the emergence of colistin hetero-resistance after patients had prolonged exposure to colistin[12, 13].

Entirely new agents against *A.baumannii* are under evaluation and development but are unlikely to be available for clinical use for many years and in the meantime clinicians have considered many ad-hoc combinations of existing licensed drugs in the hope that they will act synergistically[14, 15]. Effective combination therapy with low colistin doses has been suggested as the best strategy to counter colistin-resistant *A. baumannii*[13, 16].

Research groups recently developed robust alternatives to traditional mammalian models of infection to provide evidence of *in vivo* efficacy due to concerns about practicality, cost and ethical issues[17]. Invertebrate models have been under consideration as an inexpensive and laboratory based alternatives[18-24]. This study investigates the use of the *Galleria mellonella* invertebrate model to evaluate the effects of mono- and combination therapy against biofilm associated infections.

2 LITERATURE REVIEW

2.1 ACINETOBACTER BAUMANNII

A. baumannii had been established as one of the most clinically problematic nosocomial pathogens due to its resistance to multiple antibiotics (Multidrug-resistant, MDR), remarkable ability to up regulate and/or acquire resistant mechanisms and ability survive for prolonged periods throughout hospital environments; *A. baumannii* is increasingly becoming one of the most studied species of the genus *Acinetobacter*[5].

A. baumannii is an aerobic Gram negative, catalase positive, oxidase negative, nonmotile, nonfermenting bacterium. They are cocco-bacilli that are difficult to destain which can lead to their misidentification as either Gram negative or Gram positive[1, 5, 25].

2.1.1 Natural history and Epidemiology

2.1.1.1 Natural Habitats

Understanding how prevalent the natural habitat of *A. baumannii* is can provide a greater understanding of the infection characteristics of these bacteria however due to misreporting in earlier studies there has been confusion about *A. baumannii* and its natural habitat. Giamarellou *et al.*[1] stated that *A. baumannii* is everywhere in nature, which is certainly true in the case of certain members of the genus *Acinetobacter.* These species are considered ubiquitous organisms that are readily sourced from virtually all samples of soil or the surfaces of water[5]. However this is a misconception for *A. baumannii* species and in a review carried out by Peleg *et al.*[5] *A. baumannii* was rarely found to be on the skin or faeces of patients with

uncompromised immune systems. Moreover although it had been found in soil samples from Hong Kong and on vegetables from the UK, *A. baumannii* does not appear to be an environmental organism[5]. In conclusion, *A. baumannii* should not be considered ubiquitous, however the lack of data available from the report did mean that the natural habitats of *A. baumannii* were not able to be accurately defined and remain this way[5, 26].

2.1.1.2 Epidemiology

In Europe transitions of the epidemic strains of *A. baumannii* have been observed in hospitals with the mostly likely cause of transfer via colonised patients. The spread of Multidrug-resitant (MDR) *A. baumannii* was found not to just be confined to hospitals within a city but also on a national scale[5]. From late 2003 to 2005, The Public Health England (formerly Health Protection Agency) national reference laboratory received approximately 1600 isolates of *Acinetobacter* spp. including carbapenem-resistant *A. baumannii* species. Representative of these clones were obtained from 48 hospitals in total in southeast England, indicating the widespread of the clones across counties[27].

Most concerning is the increased reports of intercontinental spread of *A. baumannii* has also been described between Europe and other countries as a consequence of colonised patients being transferred for treatment via air travel[5 28]. The ability of *A. baumannii* to survive in the environment, together with an enhanced capacity to acquire antibiotic resistance determinants, may have contributed to the bacterium's ability for successful dissemination and persistence in hospitals across the world[26].

2.1.2 Source of infection

Over the past 20 years the significant role of *A. baumannii* in the colonisation and infection of immune compromised patients admitted to hospital has increased. As a pathogen, *A. baumannii* specifically targets moist tissues such as mucous membranes or areas of the skin that are exposed through accident or injury[26]. The bacterium has been implicated in a variety of nosocomial infections including bacteraemia, urinary tract infections, secondary meningitis and, in particular, ventilator-associated pneumonia and in the invasion of burn wounds[2, 5].

A. baumannii is an opportunistic pathogen generally effecting individual patients with an already compromised immune system; *A. baumannii* is usually non-pathogenic in healthy individuals. However Jones *et al.*[3], who investigated casualties with uncompromised immune systems from Iraq, found *A. baumannii* to be one of the major causes of bacterial wound infection. This suggests that *A. baumannii* is not only an opportunistic pathogen in individuals with a compromised immune system, but also those who have had medical invasive devices fitted, such as catheters or open wounds.

In addition to this, work by Zanetti *et al.*[7] also found that *A. baumannii* infection is not only transferred from patient to patient, but also from the environment where colonised patients had been isolated for treatment. Zanetti *et al.*[7] reported on the case of a burns victim who had contracted an *A. baumannii* infection whilst being transferred to hospital. Two other patients from the same ward were also infected, however environmental swabs samples of the ward tested negative for *A. baumannii.* Six months later, 6 patients contracted the same strain of *A. baumannii*. Environmental swab samples taken tested positive for *A. baumannii*

showing wide spread environmental contamination is possible through patient to patient contact.

2.1.3 Mechanisms of resistance

A. baumannii is defined as multidrug resistant to nearly all available antibiotics, including beta-lactams, fluoroquinolones, tetracyclines and aminoglycosides[16]. The wide array of antimicrobial resistant mechanisms that have been described for *A. baumannii* is extensive[5].

2.1.3.1 Intrinsic resistance

Intrinsic resistance is the innate ability of a bacterial species to resist the activity of particular antimicrobial agents through its structural or functional characteristics[29]. One reason for Gram-negative resistance to a large number of antibiotics is a result of the effective permeability barrier of their outer membrane. The outer membrane is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its lipopolysaccharide (LPS)-covered surface[30].

2.1.3.2 Acquired resistance

The ability for *A. baumannii* to become resistant to such a large number of antibiotics has been attributed to the bacterium's ability to accumulate antibiotic resistant mechanisms some of which include, degradation enzymes against beta-lactam antibiotics, modification enzymes against aminoglycosides, altered binding sites for quinolones and changes in the outer membrane proteins[5, 26]. The ease at which *A. baumannii* can acquire and incorporate

genetic elements and the low permeability of the outer membrane to certain antibiotics tends to explain how *A. baumannii* has become universally a multidrug-resistant bacterium[1, 4, 31, 32].

2.1.4 Biofilms

In nature, is well accepted that bacteria do not exist as planktonic (free-cell) cultures but predominantly exist by attaching to and growing upon living and inanimate surfaces[33]. Such structures are known as biofilms, and defined by Costerton *et al*.[34] as bacterial populations encapsulated within a matrix of secreted exopolymeric substances (EPS) that are attached to a surface adherent to each other and/or surfaces or interfaces. The matrix of the biofilm provides protection of the bacterial cells to physical and environmental stressors, it also provides an environment within which the cells can communicate with each other, transferring genetic information. Most biofilms rarely exist as mono-species, rather as polymicrobial multicellular communities encased within the EPS matrix[35-38]. The predisposition of biofilms to exist as functioning multicellular communities communicating between each other via cell to cell signalling known as quorum sensing, makes bacterial cells similar to other types of living cells which are capable of unicellular existence, yet preferably reside within multicellular communities[35].

Biofilms have a great significance for public health as biofilm-associated microorganisms have been shown to be implicated in numerous debilitating, and often chronic diseases; including cystic fibrosis, orthodontic diseases, sinusitis and some forms of heart disease[39-41]. They exhibit dramatically decreased susceptibility to antimicrobial agents and this susceptibility may be intrinsic or acquired[33].

2.1.4.1 Biofilm associated *A. baumannii*

The biofilm mode of existence for *A. baumannii* can be attributed to the bacterium's increased MDR, although resistance can also be attributed to many contributing factors including overuse and misuse of antibiotics in hospitals and the community[9, 10].

A recent review into the evidence of biofilms in wounds by Percival *et al.*[42] highlighted studies that detailed biofilms in wounds, the effect they have on infection and wound healing. The studies featured in the review provided evidence that biofilms reside within the chronic wound and most interestingly represent an important mechanism underlying the observed, delayed healing and infection. In a letter to the editor, Rodriguez-bano[43] detailed a study his group had undertaken exploring the biofilm forming capabilities of *A. baumannii*. In their study they found that certain strains of *A. baumannii* were capable of forming biofilms and these strains had played a role in the pathogenesis of some device-associated *A. baumannii* infections.

2.2 REVIVING OLD ANTIBIOTICS

It is the emergence of MDR nosocomial bacteria to which there is a concerning void of discovery for new antimicrobials, that is with new target sites, which has led to the revival of antibiotics whose use was once restricted based on the discovery of less toxic antibiotics. The present study, focuses on the use of antibiotics from the polymyxin family, and in particular the polycationic antibiotic, Colistin, also known as polymyxin E.

2.2.1 Polymyxin antibiotics

Polymyxins were first available in the commercial markets the 1950s but their usage was abandoned due to the high risks of renal failure, a well-known side effect of the Polymyxins family of drugs[4, 5, 31]. The family of Polymyxins were first discovered over 50 years ago then abandoned soon after due to their toxicity, they were never subjected to the rigorous development modern drugs have undergone[31].

The Polymyxin family consist of five chemically different antibiotics, A to E, of which two, Polymyxin B and E (colistin) are currently available on the clinical market; all polymyxins have a similar antibacterial spectrum and mode of action[2, 31, 44]. Colistin and Polymyxin B have been shown to be active against Gram-negative bacteria, including *A. baumannii* [15]. Colistin and Polymyxin B comprise basic polypeptide antibiotics with a side chain terminated by characteristic fatty acids, see figure 2.1.

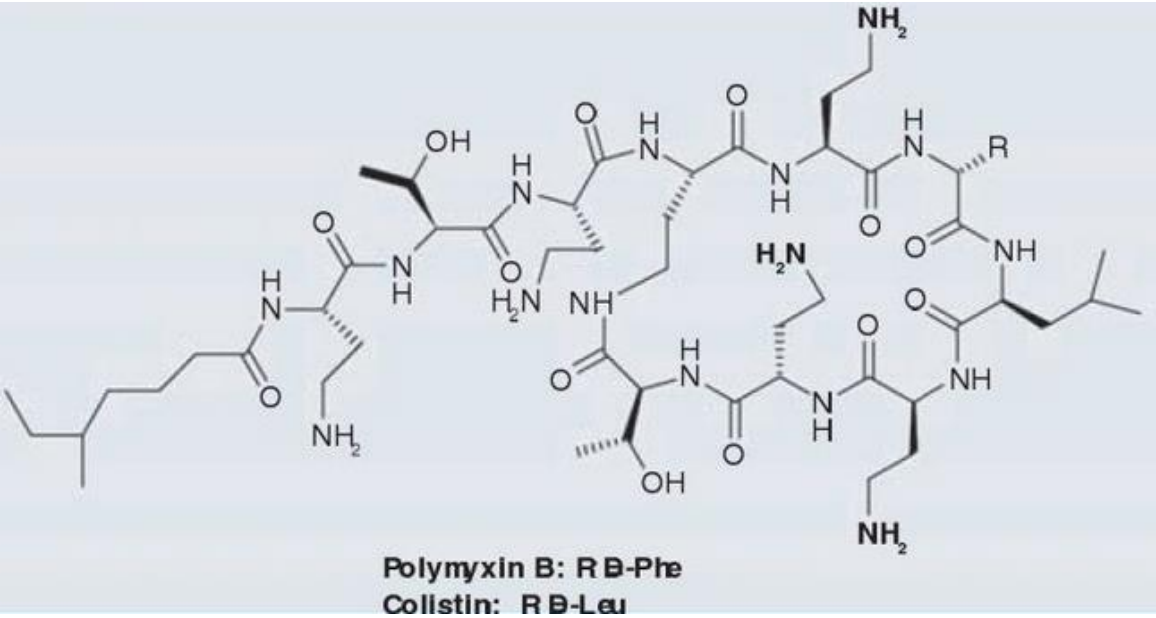


Figure 2.1: Chemical structure of Polymyxin B and E (colistin) showing the characteristic fatty acid tail. Source: Biswas *et al.* [31].

Colistin is administered as an inactive pro-drug, colistin methanesulphonate (CMS), which is converted *in vivo* to its active form, colistin. Colistin (and Polymyxin B) act by displacing magnesium and calcium (ions that normally stabilize the lipopolysaccharide molecules) from the negatively charged lipopolysaccharide in the bacterial cell membrane. This leads to a loss of integrity of the membrane and an increase in the permeability of the cell envelope, leakage of cell contents, and subsequently, cell death[45]. The initial mode of action of colistin and polymyxin B is one that is regarded as non-lethal, at low doses, both peptide compounds have been shown to disrupt and permeabilise the outer membrane without causing the death of the cell[45-47]. The bactericidal action of the polymyxins, happens after initial drug molecules bind to and disrupt the outer and inner membrane of the cell, 'free' peptide molecules then cause the leakage of the cytoplasmic contents of the cell [31, 48, 49], Figure 2.2.

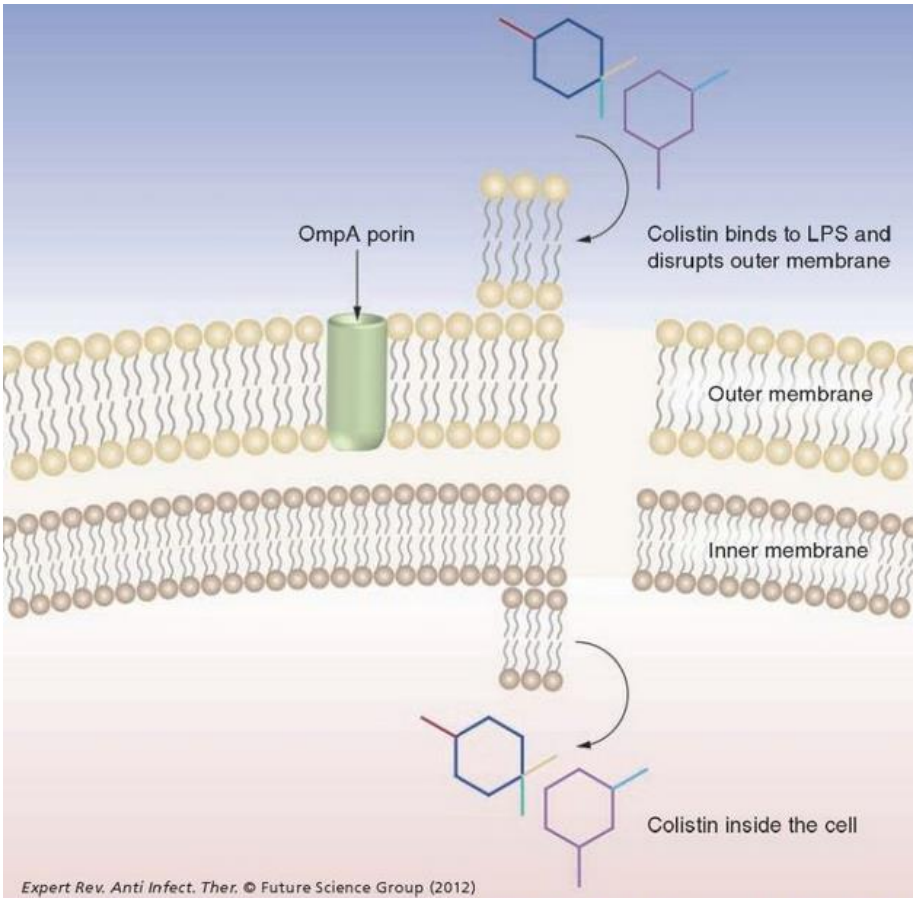


Figure 2.2: Schematic diagram of the mode of action of colistin. Source: Biswas *et al.*[31].

Recent years has seen the increased use of colistin as a last resort antibiotic for MDR Gram-negative associated infections. Figure 2.3 was taken from a review by Biswas *et al.*[31] graphically representing the number of citations collected from PubMed from dates ranging from 1960 to the middle of 2011. The graph shows the trend in popularity for the use of colistin in research, which has increased in the 21st Century. The graph also shows the increase of colistin resistance over the same time period. As the number of publications increase, so do the reports of colistin resistance.

It is also noteworthy that there has been an increase in heteroresistance of colistin by *A. baumannii.* Research from an Australia group reported heteroresistance (resistance of one particular strain towards a particular antibiotic) of Colistin in 15 of 16 clinical isolates[1, 15] causing concern that Colistin resistant *A. baumannii* may be more prevalent than expected.

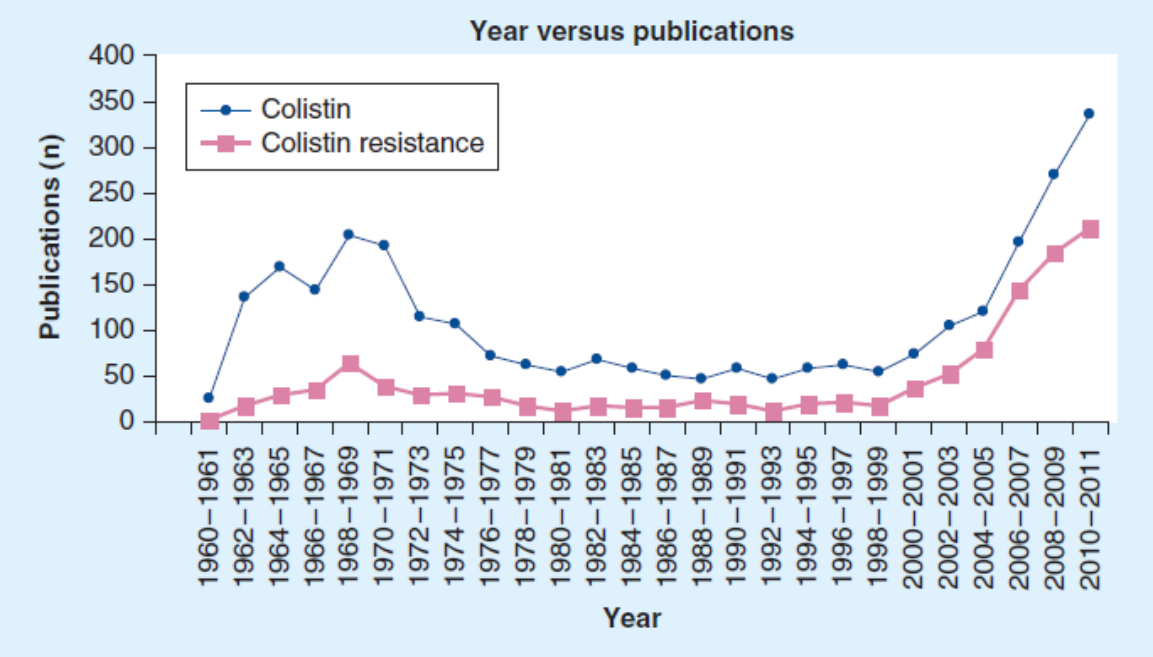


Figure 2.3: Shows the number of citations found in the PubMed database from 1960 to the middle of 2011 using either the term 'colistin' or 'colistin resistance'. Source: Biswas *et al.*[31].

This is a concern when addressing the use of Polymyxin E as a therapeutic treatment by itself and therefore researchers are looking to the possible use of combination therapies, to counteract this resistance. Hornsey *et al*.[13] researching into alternative combination therapies found that glycopeptides- Polymyxin E combination therapies are highly active against *A. baumannii* infections in simple invertebrate models of infection.

2.2.2 Other antibiotics

Some studies have suggested that certain hydrophilic antibiotics, such as rifampicin, carbapenems, glycopeptides and tetracyclines can work synergistically with colistin but this is pathogenic organism dependant [15, 16, 31]. Broad spectrum antibiotics are antibiotic that are effective in treating a wide range of infectious microorganisms rather than the restricted range of 'narrow spectrum' ones.

The selection of antimicrobials chosen for use in combination with colistin was based namely on the type of mode of action of the antibiotics within the bacterium ensuring diverse mechanisms are present. Based on this chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin and tetracycline were chosen. Gentamicin, Tetracycline, Erythromycin and Chloramphenicol were chosen as antimicrobials which disrupt protein synthesis through interactions with the ribosomes. Rifampicin was chosen as it disrupts RNA synthesis, therefore disrupting protein synthesis through a different mechanism and is bactericidal[50], Figure 2.4.

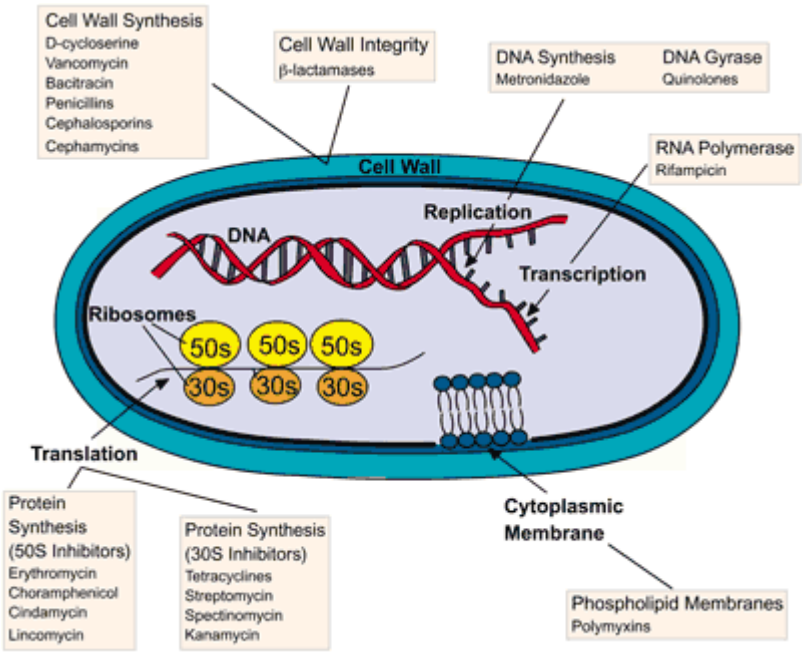


Figure 2.4: Mode of action for a variety of different bacteriostatic and bactericidal antibiotics. Image courtesy of Wiley 2004 Essential Biochemistry. Image accessed July 2014.

2.3 INFECTION HOST MODELS

To assess the efficiency of antimicrobial treatment *in vivo* models are routinely used in the pharmaceutical industry before clinical trials are undertaken. As human studies are limited to individual case reports or retrospective cohort studies, mammalian models are the usual source for *in vivo* research. However many concerns arise when using mammalian models to study antimicrobial treatment, these including practical, cost and ethical issues[13, 17]. As a result of this invertebrate models are being developed in our labs and elsewhere as an inexpensive and viable alternative to the use of mammalian models.

Although numerous cases have been reported where several mammalian opportunistic pathogens have infected insects only a few studies have investigated whether a significant

correlation exists between the virulence in insects and mammals. One explanation for the limited research on the use of invertebrates as host models could be that in previous years it was naively thought that invertebrates have minimal immune defence systems; an argument based around the idea that insects are able to rapidly reproduce offspring and therefore have no need to have an intricate immune defence system. However recent research carried out by Salzet[24] found that similar patterns existed in both vertebrate and invertebrate innate immune response when infected by opportunistic pathogens. Jander *et al.*[21] also found that a positive correlation exists between virulence of *Pseudomonas aeruginosa* in mice and insects. This supports the idea that invertebrates can be used as an alternative but still sophisticated model in antimicrobial investigation.

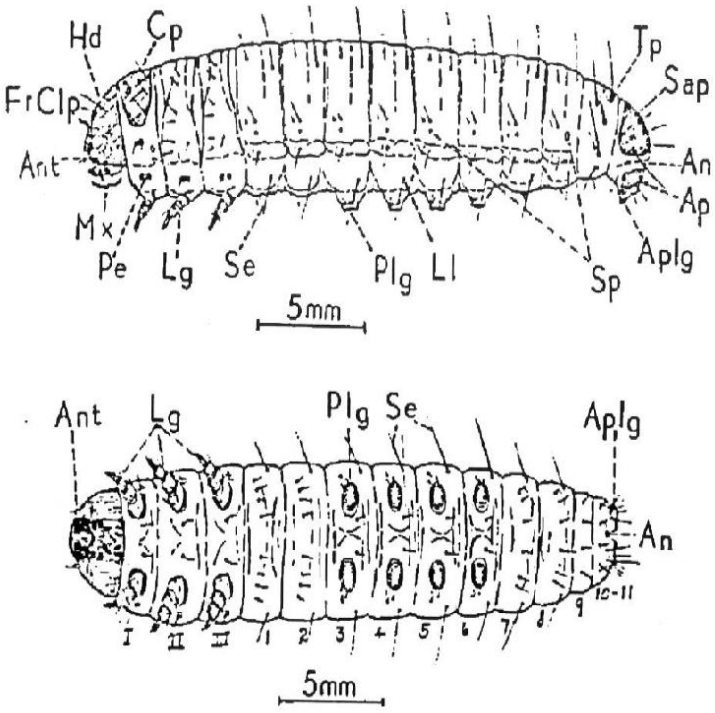
2.3.1 Galleria mellonella

Galleria mellonella, the larvae of the greater wax moth (Figure 5) is one of the most described invertebrates used as a host model to study pathogens of organisms [50]. *Pseudomonas aeruginosa,* Group A Streptococcus, *Listeria monocytogenes*, *Francisella tularensis, Acinetobacter baumannii*, *Legionella pneumophila*, pathogenic fungi and yeasts have been investigated[13, 15, 20-23, 51-57]. Interestingly, a correlation with the virulence of certain microbes in mice and *G. mellonella* has also been established[21]. *G. mellonella* is one of the most used invertebrates of choice as it has a basic immune response similar to that of mammals. *G. mellonella* immune response system consists of not only structural and passive barriers, but also cellular and humoral responses facilitated by the hemolymph system (circulatory system in *G. mellonella*)[18, 20]. The *Galleria* model has been used as both a parenteral injection model and as an oral infection model[58].

Figure 2.5: *G. mellonella* (The Greater Wax moth)

Other invertebrates have been used in the investigation of human opportunistic pathogens, such as *Drosophila melanogaster*[19], however these were found to have limitations which affected the efficiency of the model such as inability to survive at optimum temperatures of 37^{o}C. *G. mellonella* larvae were not found to have these limitations, is cheap and convenient for laboratory-based studies[22].

A schematic of the anatomy of *G. mellonella* is shown below. Most studies have used the left proleg on the underside of the larva for administration of bacteria and drugs (Figure 2.6).



Key to Abbreviations

An – Anus, **Ant** – Antenna, **Aplg** – Anal Proleg, **Ap** – Anal Plate, **Cp** – Cervical Plate, **FrClp** – Frontoclypeus, **Hd** – Head, **Lg** – Leg, **Ll** – Lateral Lobes, **Mx** – Maxillae, **Pe** – Penaculum, **Plg** – Proleg, **Sap** – Suranal Plate, **Se** – Setae, **Sp** –

Figure 2.6: Anatomy of *G. mellonella* showing the position of the proleg characterised by Smith[59].

2.3.2 Insect immune response

The innate immune system, unlike the adaptive immune system of insects and mammals, shares a high degree of structural and functional homology. As the innate immune system is the mammalians main line of defence in vertebrates, insects have become an attractive model which could potentially provide an indication of mammalian response to pathogens[17]. The cellular response of invertebrates is mediated by hemocytes and involves responses such as phagocytosis, encapsulation, and clotting[60-62].

2.3.3 Wound healing

Wound healing capabilities of *G. mellonella* larvae has been extensively studied by Rowley and Ratcliffe[63]. They found immediately after wounding both fat-body and hemolymph of the larvae were forced through the wound to form a plug. At the same time, the hemolymph coagulated forming vast networks of strand-like material attached to the hemocytes underlying the wound. After one to two hours this plug melanized and the cells became highly necrotic. Six hours after wounding, there was a massive influx of hemocytes which eventually attached to the melanized layer over the wound to form a multicellular sheath. Twelve to twenty-four hours later, the epidermal cells underlying the broken cuticle detached and migrated across the wound to form a new intact layer. This intricate wound healing process is not too dissimilar to that of the mammalian.

2.3.4 In vitro analysis of antibiotic susceptibility

2.3.4.1 Minimum inhibitory concentration

The MIC of an antibiotic against a pathogen is one of many factors that determine the best drug to cure an infection. Although, comparing the MIC of antibiotics against a target organism *in vitro* alone, clinicians may draw the erroneous conclusion that the agent with the lowest MIC against a bacterium is the preferred choice.

The following definitions of susceptibility, indifference and resistance have been defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[64], a joint organisation dealing with breakpoints, technical aspects of phenotypic *in vitro* antimicrobial susceptibility testing and functions as the breakpoint committee of the European Medicines Agency (EMA) and the European Centre for Disease Prevention and Control (ECDC).

Clinically Susceptible (S): A microorganism is defined as clinically susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success.

Clinically Indifferent (I): a micro-organism is defined as intermediate by a level of antimicrobial agent activity associated with uncertain therapeutic effect. It implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations

Clinically Resistant (R): a microorganism is defined as resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure.

The resulting values obtained via appropriate *in vitro* susceptibility testing are the clinical breakpoints, these are presented as S ≤ x mg/L; I > x , ≤ y mg/L and R > y mg/L [64].

Antibiotic	S	R
Ampicillin	-	-
Colistin	≤2 mg/L	>2 mg/L
Ciprofloxacin	≤ 1 mg/L	> 1 mg/L
Gentamicin	≤ 4 mg/L	> 4 mg/L
Erythromycin	-	-
Tetracycline	-	-
Chloramphenicol	-	-
Rifampicin	-	-
Kanamycin	-	-

Table 2.1: EUCAST MIC breakpoints for *Acinetobacter* spp. for the antibiotics to be used in this study. N.B. " - " Susceptibility testing not recommended as the species is a poor target for therapy with the drug, these drugs should be reported as resistant [64].

Ability to predict the resistance of A*. baumannii* based upon comparisons between MICs and predetermined resistance breakpoints may be less certain than other bacteria. A review paper by Perez *et al.* [65] state that due to gaps in the current knowledge of clinical response and bacterial mechanisms make existing breakpoints imperfect.

2.3.4.2 Techniques for defining MIC

Disk susceptibility testing remains the most commonly used technique in clinical microbiology laboratories; however the accuracy of colistin use in a disk diffusion assays is unsatisfactory because polymyxins diffuse poorly into agar[66]. EUCAST recommend for MIC testing of colistin against *Acinetobacter* spp. to use an alternative method to determine the breakpoints. Broth microdilution has been considered as the optimum method for determining MIC values, however this technique can be viewed as impractical and time consuming in clinical microbiological laboratories. Etests have been evaluated by both Behera *et al.* [66] and Galani *et al.*[67]. Both groups found Etests to have good concordance with broth microdilutions but poor accuracy results with disk diffusion, interestingly however, Behera *et al.* [66] suggested that the disk diffusion method could be useful for initial screening in diagnostic laboratories. In this study, MIC susceptibility will be evaluated using broth microdilutions. The synergy of antibiotics in combination will then be assessed using broth microdilution checkerboard assays.

2.4 PROJECT AIMS AND OBJECTIVES

The aim of this project is to establish whether *G. mellonella* can be used as both a biofilm associated infection and planktonic infection model to evaluate the effects of mono- and combination therapy against MDR infections.

2.2 Objectives

- Establish which/if any of the board spectrum antibiotics used in this study have a synergist effect against MDR *A. baumannii* when used in combination with colistin *in vitro.*
- To evaluate the effectiveness of the synergistic combinations against MDR *A. baumannii* in the *G. mellonella* model
- Investigate a suitable method with will enable us to study the pathogenicity of biofilm associated infections *G. mellonella*

- Establish a biofilm infection model with *Galleria mellonella* as the host species

- Study different antimicrobial agents in the treatment of biofilm grown *A. baumannii* infection in the *G. mellonella* model.

3 MATERIALS AND METHODS

3.1 *GALLERIA MELLONELLA*

For ease and convenience, *G. mellonella* were purchased from Live Foods UK. For these experiments the larvae estimated to be in the fifth instar of their life cycle, weight of the larvae varied slightly, but on average they weighed 250 g ± 25 g. Larvae were rejected if they didn't meet the weight requirements, had any skin decolourisation or obvious abnormalities. The larvae were stored in their original container with sawdust at room temperature in the microbiology laboratory at the University of Lincoln until required. The larvae were used within 4 days of purchase and required no additional food or water.

3.2 BACTERIAL STRAINS

3.2.1 Acinetobacter baumannii ATCC 19606

As mentioned before, *A. baumannii* wild type ATCC strain 19606 was purchased as a cultiloop from Oxoid LTD, UK (CL1007). The Cultiloops were stored at 5^{o}C in a fridge in the microbiology laboratory at the University of Lincoln until they were required. To culture from the cultiloops, working aseptically, one of the disposable inoculation loops containing the preserved stabilised microorganism that has been derived from ATCC 19606 was removed from the sterile packaging and carefully broken into a sterile glass vial containing 10ml of autoclaved LB broth. This inoculum was then incubated statically for 1 hour at 37^{0}C, this culture was labelled as the original culture. After 1 hour, a sub-culture was made by aseptically transferring 1ml of the original culture into 9 ml of sterile LB broth, this was labelled the working culture. Both cultures were then incubated at 37^{o}C overnight.

3.2.2 A. baumannii Clinical strains

Other strains of *A. baumannii* used in this study were gifted from Public Health England, Porton Down, UK. The strains included the characterised strain AYE and were received as glycerol stocks.

3.2.3 Escherichia coli ATCC 25922

Like *A. baumannii* ATCC 19606, *E. coli* ATCC 25922 was also purchased as a culti loop from American Type Culture Collection strains, Oxoid LTD, UK. Both ATCC 19606 and 25922 are defined as wild type micro-organisms; that is a strain of a species which has the absence of acquired and mutational resistance mechanisms to the drug in question as defined by EUCAST.

3.2.4 Culturing medium

Although several selective and differential media are available for culturing of specific *Acinetobacter* and *Escherichia* species, both can be cultured readily on routinely used laboratory mediums, such as sheep blood agar (BA), tryptic soy agar (TSA) and Luria-Bertoni (LB) medium at a 37^{o}C incubation temperature overnight[5]. In this study LB broth and agar was used exclusively for the culturing and maintenance on streak plates and slopes of all strains used. To produce LB broth, LB power (1280-1660, Fisher Scientific, UK) 20g was suspended in one litre of distilled water, table 3.1.

For the preparation of agar plates, agar (1.5%w/v) was included in the powder mix. The solution was then dissolved by heating with frequent agitation, sterilised in autoclave 121ºC for 15 minutes then allowed to cool to 45-50ºC, before mixing and then dispensing into plates. Tryptone and Yeast extract (YE) serve as a source of nitrogen, sulphur and carbon

while YE also contains Vitamin B complex. Sodium chloride provides sodium ions for the membrane transport and maintains osmotic equilibrium of the medium.

Formula (g/L)	
Tryptone	10
Yeast Extract	5
Sodium Chloride	5
Agar*	15
Final pH 7.2 at 25^{o}C	

Table 3.1: Formula for the preparation of Luria Bertoni (LB) medium. *Agar should only be added for the preparation of agar plates, and excluded when making broth.

For experiments where the need to distinguish between the two different species, *A. baumannii* and *E. coli*, was required, Membrane Lauryl Sulphate Agar (MLSA) was used. MLSA is a selective media type used in for the presumptive detection of coliforms. Coliform groups are both aerobic and anaerobic facultative, Gram-negative, non-spore forming rods which ferment lactose producing acid at 37^{o}C. On MLSA plates, *E. coli* appear as yellow colonies as they belong to the Coliform group whereas *A. baumannii* colonies appear as pink colonies. To produce MLSA plates, 92.2 grams of the medium were suspended in one litre of distilled water, see table 3.2 The solution was then dissolve by heating with frequent agitation,

sterilised in autoclave 121ºC for 15 minutes then allowed to cool to 45-50ºC, before mixing and then dispensing into plates.

Formula (g/L)	
Casein Peptone	40.00
Lactose	30.00
Yeast Extract	6.00
Sodium Lauryl Sulphate	1.00
Phenol Red	0.20
Bacteriological Agar	15.00
Final pH 7.4 ± 0.2 at 25^{o}C	

Table 3.2: Formula for the preparation of Membrane Lauryl Sulphate Agar (MLSA)

3.2.5 Maintenance of cultures

To maintain a healthy culture of the bacteria throughout this study and also to enable future research 2 different stock culturing techniques were employed, cultures streaked on agar plates are not considered stock cultures.

The first one which preserves the culture indefinitely if stored correctly is known as glycerol stock. To prepare this culture, 100 ml of 10% glycerol-LB broth was prepared and autoclaved sterile. 500 µl of a fresh overnight culture of bacteria was then transferred to a sterile 1.5ml Eppendorf to which 500 µl of the glycerol stock. Six Eppendorfs were prepared, 3 were stored at -20^{o}C and the other 3 were stored at -80^{o}C.

The second culture technique is known as preservation by slopes. To prepare agar slopes, agar which has been gently brought to boil to allow for the granules to dissolve, is aliquoted out into glass universals then autoclaved. The glass vials are then allowed to cool on a slant to produce a sloped agar bed. A single colony is then aseptically taken of an agar plate and streaked on to the sloped agar bed in a zigzag pattern. The slopes were then stored on the bench top at room temperature. The slope remade every 3 months from fresh cultures. 2 slopes were made each time, original and working, the original slope were only used if there were signs of contamination in the working slope.

3.2.6 Preparation of bacteria for injection

In order to prepare the bacteria for injection in the *G. mellonella* model, the bacteria were suspended in to an appropriate solution for injection, a saline. This is because the bacteria cannot be injected directly from an overnight suspension in LB broth as this could cause the larva body system to go into shock and result in death. The chosen saline for this study is phosphate buffer saline (PBS), a common buffer saline used in biological research. The osmolality and ion concentration of PBS matches that of the human body. PBS was purchased from Sigma-Aldrich, UK (P5368).

1ml of an overnight culture (OD ~1.8) was aseptically transferred in to a sterile 1.5ml Eppendorf. The culture was then centrifuged at 11, 000 xg for 5 minutes, a sufficient speed and duration to spin cells in to a pellet at the bottom of the tube. The supernatant was discarded and the pellet was resuspended in 1ml sterile PBS. This process was then repeated

so that the pellet was subjected to 2 washes with PBS, then resuspended one last time in 1ml of sterile PBS.

3.2.7 Preparation, handling and storage of antibiotics

Antibiotics were purchased from Sigma-Aldrich® and prepared in stocks, filtered and stored in 500 µl aliquots in the freezer. 10 ml working stocks of each antibiotics were prepared, see table 10. To produce initial stock solutions, erythromycin, tetracycline and chloramphenicol were dissolved in 100% ethanol and rifampicin was dissolved in dimethyl sulfoxide. All other antibiotics were dissolved in sterile dH_2O; Further stock dilutions were made with sterile dH_2O, see table 3.3.

Antibiotic	Stock concentration	Volume required in ml to produce 1024 mg/L working stock (µl)
Ampicillin	100 g/L	102.4
Colistin	10 g/L	1024
Chloramphenicol	50 g/L	2048
Ciprofloxacin	10 g/L	1024
Erythromycin	10 g/L	1024
Kanamycin	50 g/L	2048
Rifampicin	50 g/L	2048
Tetracyline	50 g/L	2048

Table 3.3: Volume required to produce 1ml of 1024 mg/L working stock of the antibiotics used in this study.

3.2.8 Statistical analysis

Kaplan Meier survival/failure statistical analysis estimates the survival function from lifetime data and was used in this study to measure the percentage survival of the *G. mellonella* groups over a 96 hour period after given treatment. The significance of the survival the survival curves are analysed by Log-rank test which evaluates the equality of the survival distributions

between groups (Chi-squared). If the difference between the groups is greater than or equal to 0.05 (P=/<0.05), at a confidence limit of 95%, the groups were determined as significantly different. SPSS Statisical Analysis software 19 was used. Comparison of the mean percentage survival of the larva at the end of the 96 hour test period was made by using one tailed, unpaired t test (P = <0.05) performed using Microsoft Excel 2010.

4 ESTABLISHING PATHOGENICITY

4.1 ESTABLISHING THE MODEL

4.1.1 Life cycle of G. mellonella

Before any experiments on the larvae were carried out it was important to know whether the larva purchased from Live Foods UK were of the appropriate development stage (instar). As mentioned before the larvae selected for use weighed around 250 mg. 16 larvae, which had been subjected to no manipulation, were incubated at 37^{o}C for 96 hours. Observations were made of the survival of the larvae and also whether there had been any metamorphic changes.

4.1.2 Total Viable Counts

In order to enumerate the quantity of bacteria injected in to the larva, bacteria from an overnight culture was prepared for injection as described above in section 3.1.4 and log dilutions were made as described in section 3.2.1 and was repeated until 8 log dilutions had been prepared. 100 µl of each dilution was then spread plated on LB agar and incubated at 37^{o}C for 24 hours. Plates were counted the following day.

4.1.3 Pathogenicity of A. baumannii ATCC 19606

The pathogenicity of *A. baumannii* was established by injecting larvae in groups of 16 with 10 µl of different log dilutions produced from overnight planktonic cultures of ATCC 19606. The injections were administered into the top left proleg of each larvae. The 4 log dilutions (10^{0}, 10^{1}, 10^{2} and 10^{4}) were used to establish the pathogenicity of *A. baumannii* in *G. mellonella*, bacteria from an overnight culture was prepared for injection as described above in section 3.1.4. Log dilutions were then made by aseptically transferring 100 µl of the washed bacterial

into a sterile Eppendorf which contained 900 µl PBS, the suspension was mixed by gently pipetting up and down three times. 100 µl of this dilution was then transferred to a new Eppendorf containing 900 µl PBS. This process was repeated to produce 4 log dilutions. 16 larvae made up each of the test group, a positive control, where larvae were injected with 10 µl of PBS. A negative control group, a non-injection group, was also included. The larvae were incubated at a temperature of 37^{o}C and to ensure the environment had sufficient moisture in the air, a bowl of tap water was placed on the bottom shelf. The mortality rate of each larva was recorded every 24 hours for 96 hours, larvae were determined as dead if they failed to respond to touch. Experimental groups were repeated 3 times, each on separate occasions.

4.1.4 Kill Kinetics of bacterial growth in vivo.

To determine the kill kinetics of bacterial growth *in vivo*, larvae were injected with a lethal dose of *A. baumanni* ATCC 19606 (10^6 CFU/ml), previously described in the method in section 3.2.2. Every 2 hours individual wax worms were homogenised by grinding using a glass rod in 1 ml sterile PBS. Eight log dilutions of the homogenised solutions were then prepared and 100 µl of each dilution was then spread plated on LB agar. Plates were incubated at 37^{o}C for 24 hours. Plates were counted the following day. LB plates had been supplemented with 100 mg ampicillin to ensure only the growth of *A. baumannii* colonies. Control plates were included; Plates with no supplement and spread plates of *A. baumannii* ATCC 19606 diluted in PBS. Gram stains were also performed on random colonies from each plates to check the bacterial morphology was that of *A. baumannii.*

4.1.5 Assessing the model against clinical strains of A. baumannii

To assess the model against clinical strains of *A. baumannii* bacteria from an overnight culture was prepared for injection as described above in section 3.1.4 and log dilutions were made as described in section 3.2.1. The model was tested against clinical strains of *A. baumannii (*AYE, W, A13, A14, and A17) to assess the pathogenicity of the clinical strains against the larva. Whereas ATCC 19606, a wild type strain, does not possess the resistant mechanisms potentially developed by the clinical strains.

4.2 RESULTS AND DISCUSSION

4.2.1 Life cycle of G. mellonella

Each stadium is characterized by the slippage of the head capsule of larvae. When larvae reach the last stage before pupation, they stop feeding and start building a light silk cocoon[68]. During the incubation time of the larvae which have received no manipulation, no deaths were observed. At the end of the 4 days there was evidence that the larva had chewed on the filter paper lining the petri dishes and in some cases larva had begun to spin a fine white nest-like structure, characteristic of the later larval stages. During the final larval stages, the larva spins itself a white silken cocoon. After this, the larva enters a quiescent period in which it is transformed into a pupa[59]. None of the larvae showed any signs of reaching this stage within the 96 hour observation period. When larvae were left for a further 3 days, they began to show signs of entering the final larval stage, i.e. formed a white silken cocoon. Based on these results and the life cycle stages of Greater wax moth[68] the larvae were estimated to be in fifth instar of the larval stage.

A negative control group, one where individuals in the group had received no manipulation, was included in each following experiment to ensure larval stage consistency and also to monitor the environment.

4.2.2 Total Viable counts

In order to enumerate viable bacterial colonies, an assumption is made that each bacterial colony when immobilised on an agar plate arose from one living (viable) cell, therefore it is possible to determine the number of live bacteria defined as colony forming units (CFU) per ml of the original culture. The general acceptance range for counting colony forming units on agar plates is 30 to 300 colonies. In this study, dilution 10^{-5} fell within the acceptance range of 237 colony forming units grown on the agar plate.

The CFU/ml of the original culture can be calculated using the following formula:

CFU/ml = number of colonies per ml plated/Total dilution factor

A log dilution of 10^{-5} showed 237 colonies~~,~~ however only 100 µl of the dilution was plated, therefore the number of colonies must be divided by 0.1 ml before being divided by the total dilution factor.

Therefore;

CFU/ml = (237/0.1ml)/10^{-5} = 2370 x 10^{5} = **2.37×10^{8} CFU/ml**

The above calculation determines the CFU/ml; however the larvae were only injected with 10 µl of culture, a dilution of 10^{-2}. Therefore it is possible to determine that 10 µl of the original culture contained 2.37 x 10^6 CFU. From this the dilutions injected into the larvae have been calculated, see table 3.

Dilution	CFU/Larva (Amount injected in 10µl)
10^0	2.37 x 10^6
10^{-1}	2.37 x 10^5
10^{-2}	2.37 x 10^4
10^{-3}	2.37 x 10^3

Table 4.1: CFU/Larvae per 10 µl injections, calculated from the original CFU counts.

4.2.3 Establishing pathogenicity of A. baumannii ATCC 19606

Previous studies have shown melanisation to be an important marker of the insect innate immune system. In a recent study by Wand *et al.*[69] they found melanisation to not only to be a marker of an insect's innate immune system, but they also found a strong correlation between the ability for melanin to proliferate within the Galleria as a marker of immune evasion and survival.

In the present study this was also found to be the case, as the melanisation of the wax larva increased across the body and also darken, more likely the larva would not survive the 96 hour observation period. Melanisation of the larva is a key marker of *G. mellonella* innate immune response, Figure 7.

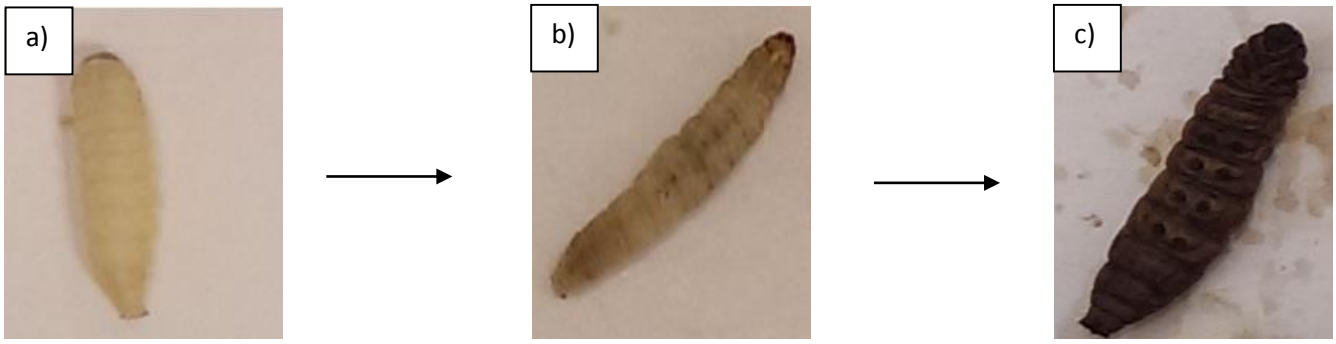


Figure 4.1: *Galleria mellonella* infection model a) A healthy caterpillar prior to injection. b) Infected larvae. c) Subsequent death of the caterpillar with a high level of melanisation and hardening.

After 96 hours, there was a greater survival by larval groups that had been infected with planktonic cultures of 10^3 CFU/larva (93.8%) compared with the groups that had been infected with 10^4 (18.8%), 10^5 (6.3%) and 10^6 CFU/Larva (0%), see figure 8. Within 24 hours post inoculation, only the group that had received an injection of PBS had 100% survival rate. For the group challenged with an injection of 10^6 CFU/ml, none had survived after 24 hours. Overall, *A. baumannii* was found to be pathogenic towards *G. mellonella* larvae when challenged with levels of 10^4 CFU or greater.

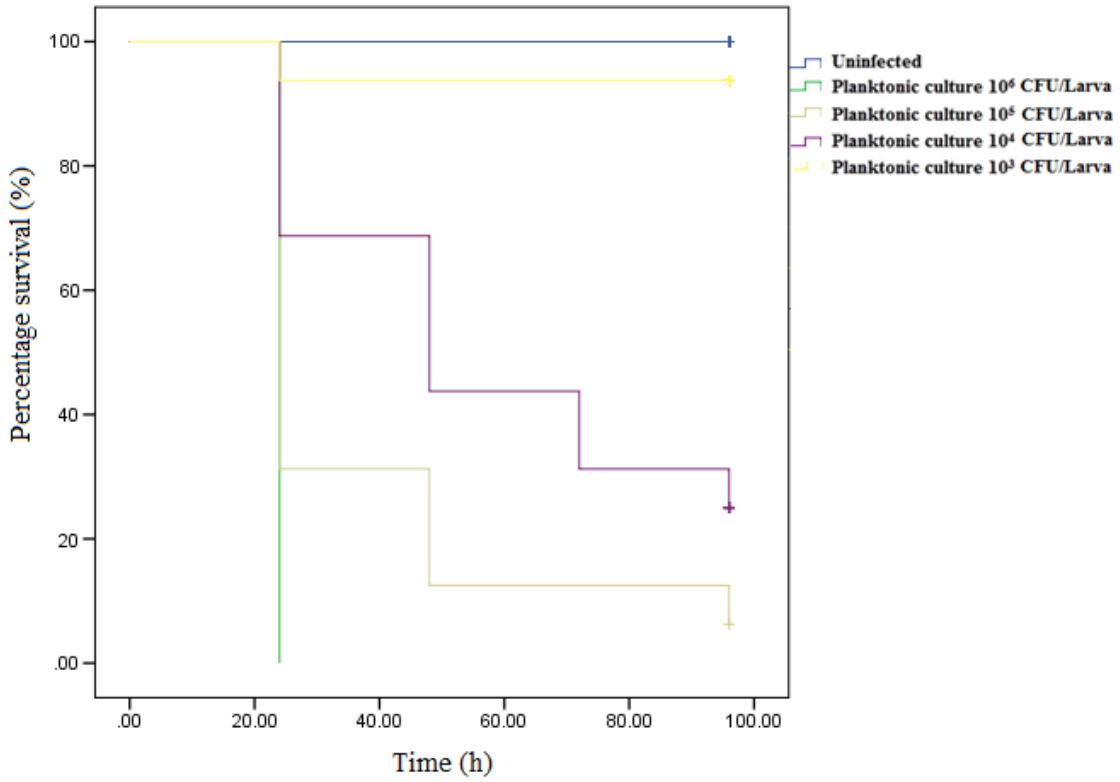


Figure 4.2: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *Acinetobacter baumannii* ATCC 19606 over a 96 hour test period.

The statistical comparison of the pathogenicity groups tested show a high level of significance, P<0.05, meaning *A. baumannii* is increasingly virulent towards *G. mellonella* wax moth larvae in a dose response manner, the higher the CFU injected into the larvae the greater the pathogenicity.

4.2.4 Kill kinetics of ATCC 19606

To ensure that the bacteria recovered from the larvae was *A. baumannii* ATCC 19606 the agar plates were supplemented with 100 mg ampicillin. In the absence of an *A. baumannii* infection, there was a baseline level of natural flora recovered from the larvae which was able to grow on LB plates, but not on LB plates supplemented with 100 mg ampicillin. *A. baumannii* ATCC 19606 grew well on the ampicillin supplemented plates.

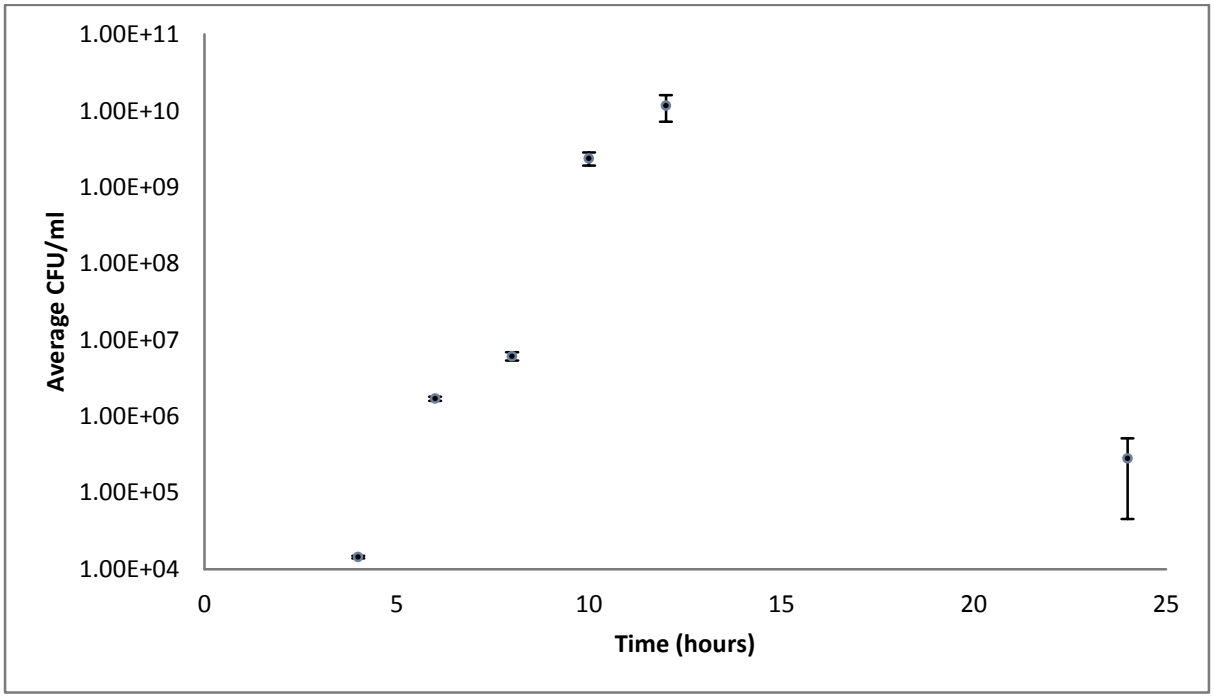


Figure 4.3: Kinetics of *A. baumannii* ATCC 19606 bacterial growth within the *G. mellonella* infection model. After initial inoculation, samples were taken at specific time points and homogenised in 1 ml PBS and plated on 100mg ampicillin supplemented LB agar plates. Average CFU counts have been presented, the error bars represent the standard error measurement (±SE).

As expected, bacterial numbers increased over time until reaching what seemed to be a critical value which caused the death of the wax worm, figure 4.3. 4 hours post inoculation, the larva infected with *A. baumannii* ATCC 19606, had begun to show signs of melanisation (Figure 4.4c). The larvae however were still active and behaving in their usual manner. In comparison, the control groups injected with PBS showed no signs of melanisation (Figure 10 b). When plated there was no bacterial growth from the homogenised control groups. The CFU at this time point averaged at 1.44 x 10^4 CFU/larva (+/- 5.9 x 10^2 SE), see figure 4.4.

Figure 4.4: 4 hours post inoculation a) *A. baumannii* infected control group. B) PBS control. C) Kill kinetic group.

6 hours post inoculation the larva infected with *A. baumannii* ATCC 19606, were beginning to move in a sluggish manner and began to show signs of melanisation (Figure 4.5c). The control groups injected with PBS still showed no signs of melanisation (Figure 4.5b). When plated there was no bacterial growth from the homogenised control groups. The CFU at this time point averaged at 1.70 x 10^6 CFU/larva (+/- 9.94 x 10^4 SE), a 100 fold greater than the CFU

value 2 hours before. SE bars show confidence in the repeated groups that there is little variance in the colony counts recovered, see figure 4.3.

Figure 4.5: 6 hours post inoculation a) *A. baumannii* infected control group. B) PBS control. C) Kill kinetic group.

8 hours post inoculation there is little change in the behaviour and the degree of melanisation of the larvae. The control groups injected with PBS still showed no signs of melanisation (Figure 4.6b). When plated there was no bacterial growth from the homogenised control groups. The CFU at this time point averaged at 6.14×10^6 CFU/larva (+/- 7.73×10^5 SE), approximately triple the CFU value 2 hours before. SE bars show confidence in the repeated groups that there is little variance in the colony counts recovered, see figure 4.3.

Figure 4.6: 8 hours post inoculation a) *A. baumannii* infected control group. B) PBS control. C) Kill kinetic group.

10 hours post inoculation, 2 of the larvae failed to respond to touch after 1 minute of gentle agitation. The control groups injected with PBS still showed no signs of melanisation (Figure 4.7b) and when plated there was no bacterial growth from the homogenised control groups. The CFU at this time point averaged at 2.39 x 10^9 CFU/larva (+/- 4.67 x 10^8 SE), approximately a 1000 fold greater than the CFU value 2 hours before. SE bars show confidence in the repeated groups that there is little variance in the colony counts recovered, see figure 4.3.

Figure 4.7: 8 hours post inoculation a) *A. baumannii* infected control group. B) PBS control. C) Kill kinetic group.

By 12 hours post infection none of the larvae were responding to touch and therefore were declared dead. The control groups injected with PBS still showed no signs of melanisation and when plated there was no bacterial growth from the homogenised control groups. The CFU at this time point averaged at 1.16 x 10^{10} CFU/larva (+/- 4.44 x 10^{9} SE), approximately a 10 fold greater than the CFU value 2 hours before (Figure 4.3). Between 10 and 12 hours post inoculation death of the larvae occurs, which means it is possible to determine that the critical value at which death of the larvae occurs around 10^{9} CFU/Larva.

24 hours post inoculation, all of the larvae had been dead for at least 12 hours. Just by looking at the graph there is an obvious decline in viable cell numbers (Figure 4.3). The error bars above and below the mean value show there to be a much wider distribution of the original data values which tells us at this point, the samples taken were much more varied. One explanation for this could be due to the variability in nutritional resources available to sustain the bacteria. Another explanation for the decline in numbers could be the changing environment upon the death of the larva, i.e. potentially competing bacteria that begin to

colonise the body of the dead larva. However the process and rate at which the larvae begin to decompose, as far as the author is aware, is unknown. What is important here is to understand that the bacteria were still able to survive within the wax worms for at least another 12 hours after the larva were noted as dead.

4.2.5 Assessing the model with clinical strains of A. baumannii

Assessing clinical strains in the model is interesting to investigate how different strains respond in the model. Such strains may possess different virulent genes which would impact the strains pathogenicity towards the larva, and the effect the possible treatment envisaged in the clinic.

In the present study a range of the following clinical strains gifted from Public Health England were assessed in the *G. mellonella* model.

Clinical Strain: AYE

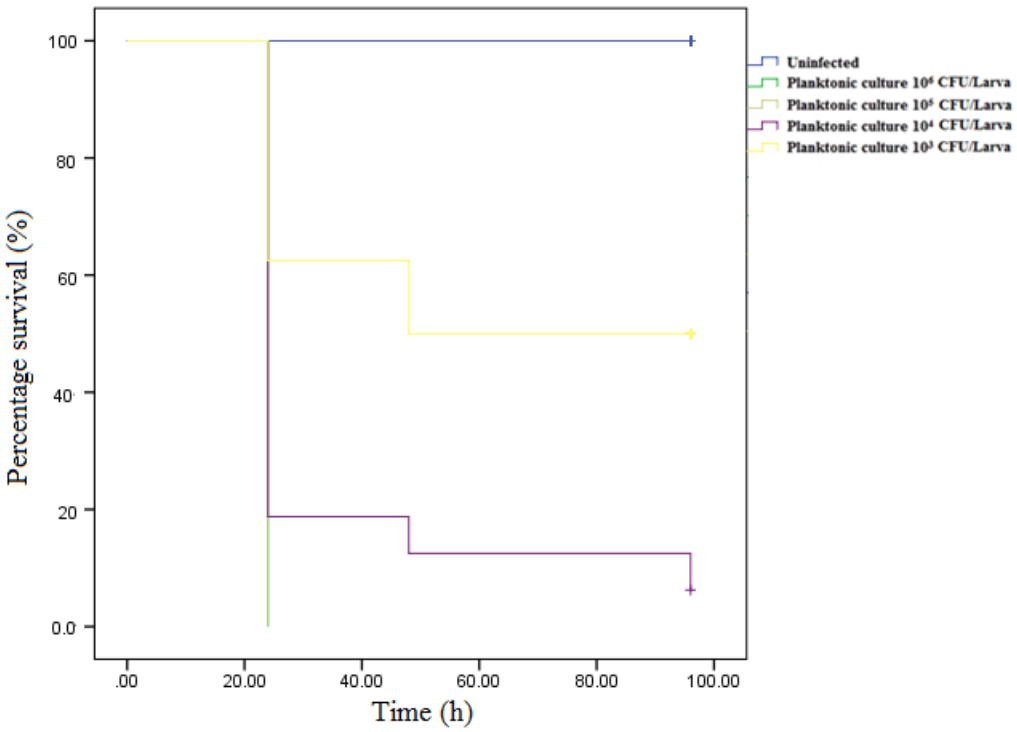


Figure 4.8: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *A. baumannii* clinical strain AYE over a 96 hour test period.

After 96 hours, there was a greater survival by larval groups that had been infected with planktonic cultures of 10^3 CFU/larva (50.0%) compared with the groups that had been infected with 10^4 (6.3%), 10^5 (0%) and 10^6 CFU/Larva (0%), see figure 14. Within 24 hours post inoculation, only the group that had received an injection of PBS had 100% survival rate. For the group challenged injections of either 10^5 or 10^6 CFU/ml had 0% survival after 24 hours.

Overall, Strain AYE was found to be pathogenic towards *G. mellonella* larvae when challenged with levels of only 10^4 CFU or greater.

Clinical Strain: W

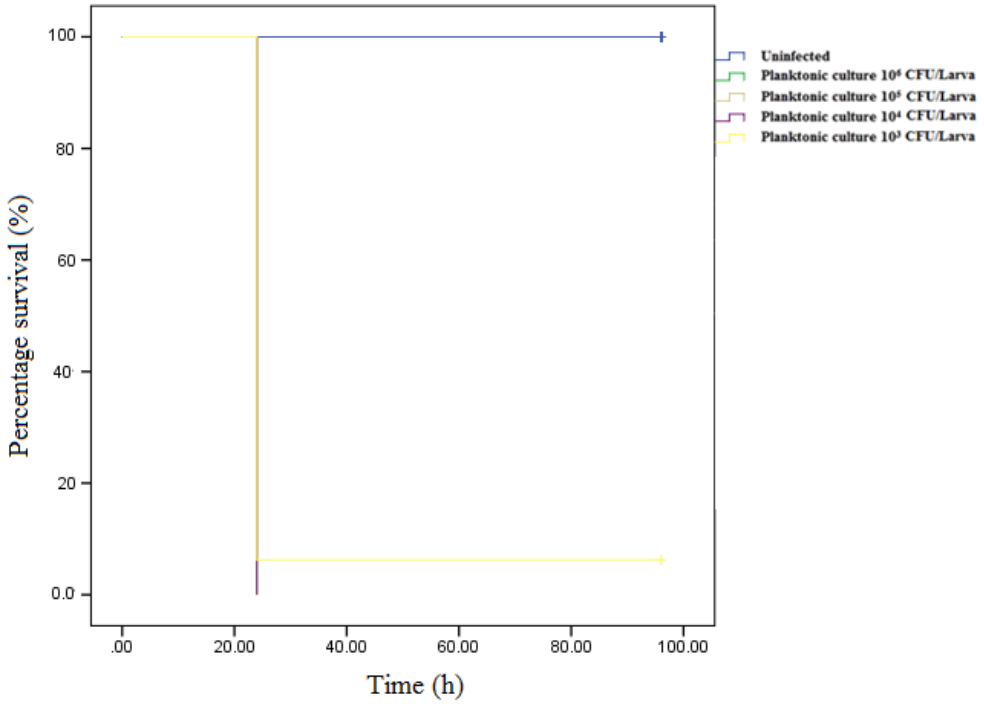


Figure 4.9: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *A. baumannii* clinical strain W over a 96 hour test period.

Strain W was highly pathogenic towards the larval groups. Groups infected with planktonic cultures of 10^3 and 10^4 CFU/larva had a survival rate of 6.3% after 96 hours post inoculation.

Groups injected with 10^5 and 10^6 had 0% survival after 24 hours. Overall, Strain W was found to be pathogenic towards *G. mellonella* larvae when challenged with levels of 10^3 CFU or greater.

Clinical Strain: A2

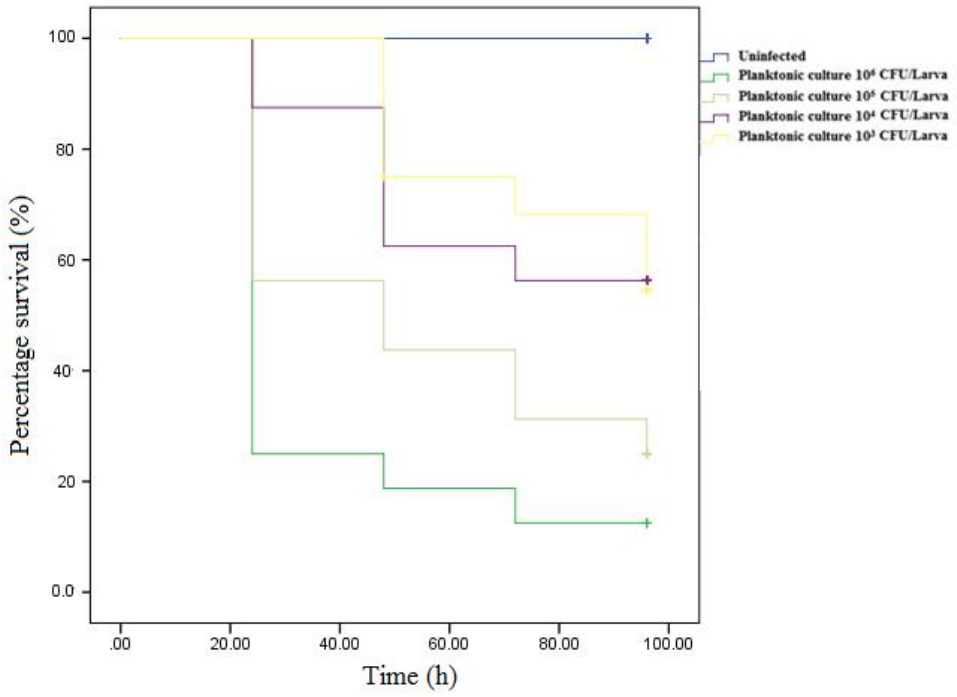


Figure 4.10: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *A. baumannii* clinical strain A2 over a 96 hour test period.

After 96 hours, there was a greater survival by larval groups that had been challenged with doses of 10^3 and 10^4 CFU/larva (56.3%) compared with the groups that had been infected with 10^5 (25.0%) and 10^6 CFU/Larva (12.5%), see figure 16. Groups that had received an injection of PBS had 100% survival rate. Overall, Strain A2 was found to be the least pathogenic strain to have infected *G. mellonella* larvae, however the statistical comparison of the pathogenicity groups tested show a high level of significance, P = 0.01, meaning strain A2 is increasingly virulent towards *G. mellonella* wax moth larvae in a dose response manor, the higher the CFU injected into the larvae the greater the pathogenicity is.

Clinical Strain: A13

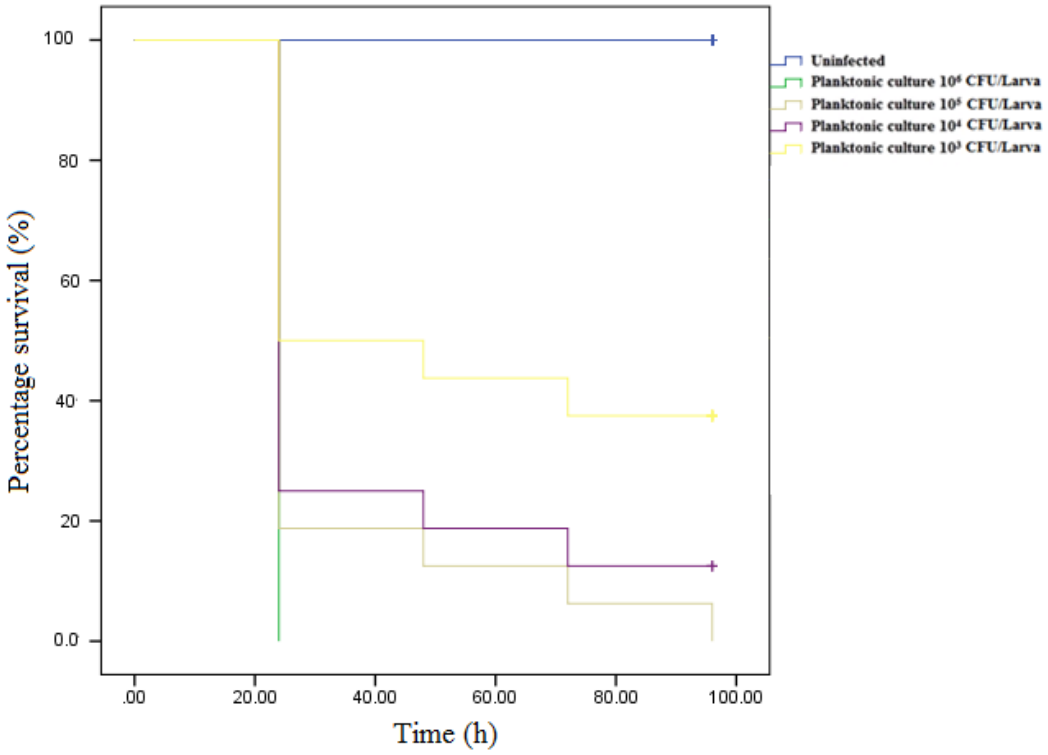


Figure 4.11: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *Acinetobacter baumannii* clinical strain A13 over a 96 hour test period.

After 96 hours, there was a greater survival by larval groups that had been challenged with doses of 10^3 CFU/larva (37.5%) compared with the groups that had been infected with 10^4 (12.5%), 10^5 (0%) and 10^6 CFU/Larva (0%), see figure 17. Within 24 hours post inoculation, only the group that had received an injection of PBS had 100% survival rate. For the group challenged injections of 10^6 CFU/ml had 0% survival after 24 hours. Overall, Strain A13 was

found to be pathogenic towards *G. mellonella* larvae when challenged with levels of 10^3 CFU or greater.

Clinical Strain: A14

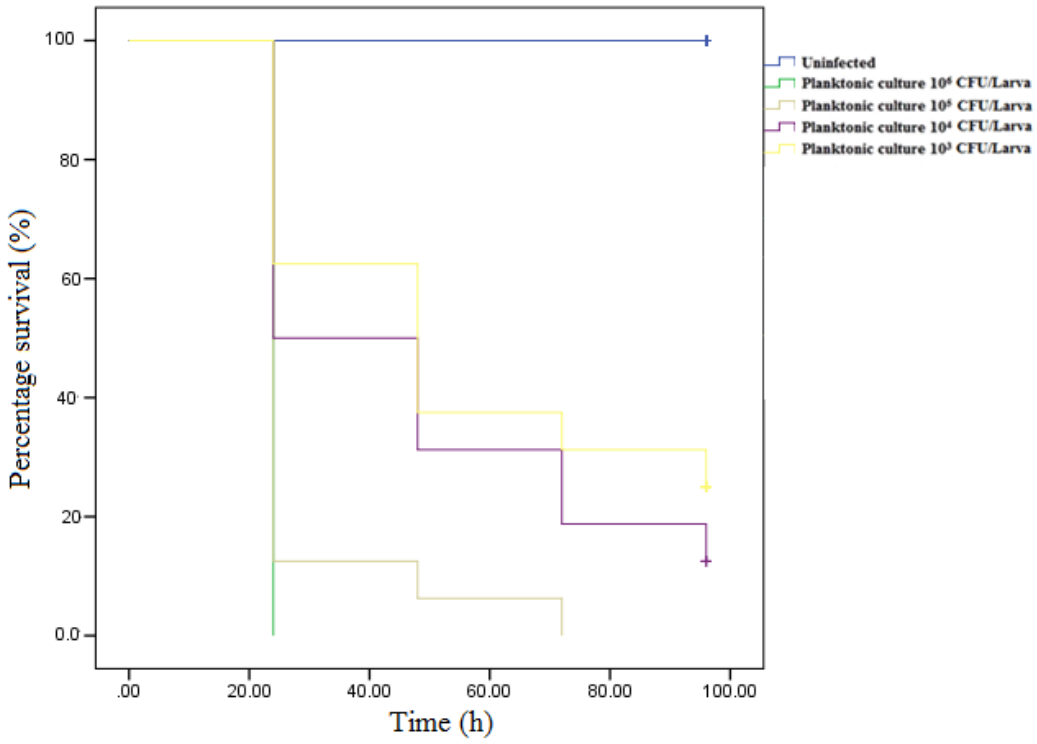


Figure 4.12: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *Acinetobacter baumannii* clinical strain A14 over a 96 hour test period.

Strain A14 was found to be highly pathogenic towards *G. mellonella.* After 96 hours, there was a greater survival by larval groups that had been challenged with doses of 10^3 CFU/larva

(25.0%) compared with the groups that had been infected with 10^4 (12.5%), 10^5 (0%) and 10^6 CFU/Larva (0%), see figure 18. Within 24 hours post inoculation, only the group that had received an injection of PBS had 100% survival rate. For the group challenged injections of 10^6 CFU/ml had 0% survival after 24 hours. Overall, Strain A14 was found to be pathogenic towards *G. mellonella* larvae when challenged with levels of 10^3 CFU or greater.

Clinical Strain: A17

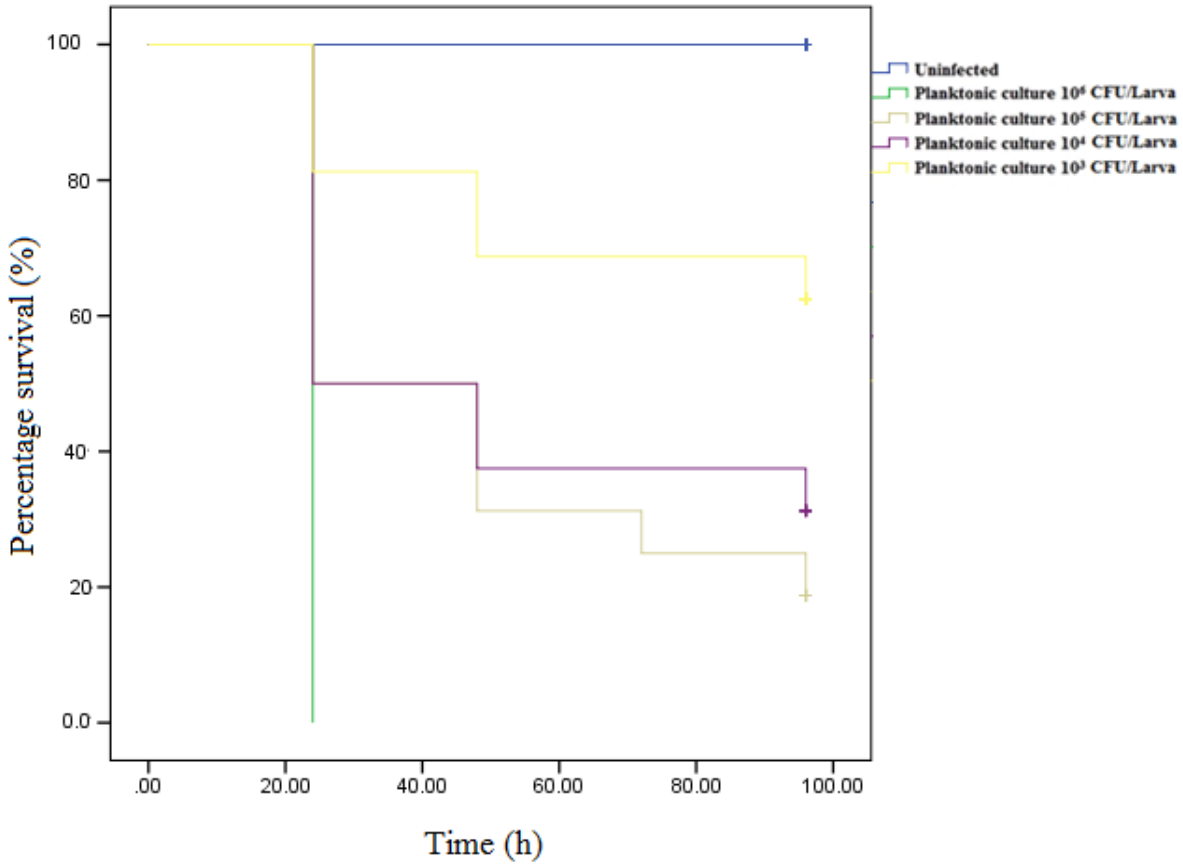


Figure 4.13: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) of *Acinetobacter baumannii* clinical strain A17 over a 96 hour test period.

After 96 hours, there was a greater survival by larval groups that had been challenged with doses of 10^3 CFU/larva (62.5%) compared with the groups that had been infected with 10^4 (31.3%), 10^5 (18.8%) and 10^6 CFU/Larva (0%), see figure 19. Within 24 hours post inoculation, only the group that had received an injection of PBS had 100% survival rate. For the group

challenged injections of 10^6 CFU/ml had 0% survival after 24 hours. Overall, Strain A17 was found to be pathogenic towards *G. mellonella* larvae when challenged with levels of 10^4 CFU or greater.

Overall, The statistical comparison of the pathogenicity groups tested show a high level of significance, $P < 0.05$, meaning *A. baumannii* is increasingly virulent towards *G. mellonella* wax moth larvae in a dose response manner, the higher the CFU injected into the larvae the greater the pathogenicity.

4.3 Summary of Results

As expected from the kill kinetics experiment focusing on *A. baumannii* ATCC 19606, the bacterial numbers increase over time until reaching a critical value between 10^{11} and 10^{12} CFU at which point causes death of the larvae, this happened within 12 hours after initial inoculation.

Planktonic cultures of all strains were found to be increasingly virulent towards *G. mellonella* in a dose response manner. That is the more CFU injected into the larvae, the increased likelihood of death. *A. baumannii* ATCC 19606 was found to be pathogenic towards *G. mellonella* at 10^4 CFU/ml. The clinical strains were similarly pathogenic towards *G. mellonella.* These results support the idea that the wax moth larva model could be a useful tool in studying the pathogenicity of problematic pathogenic bacteria.

5 *IN VITRO* SUSCEPTIBILITY AND ANTIMICROBIAL SYNERGY AGAINST PLANKTONIC *A. BAUMANNII*

5.1 INTRODUCTION

MIC assays show the minimum concentration of antibiotic required to result in no growth of bacteria. The MIC is important in detecting possible drug resistance in pathogenic bacteria and to assure susceptibility to the drugs of choice for particular infections[70].

The MIC in this study was defined as the lowest concentration of antibiotic that completely inhibited the growth of colonies detected by the naked eye. MIC values needed to be determined before the checkerboard MIC assay could be performed. Concentrations of antibiotics for the checkerboard assay need to range from four to eight times the expected MIC, to at least 1/8 to 1/16 the expected MIC in the final panel in order to observe the occurrence and magnitude of synergy or antagonism.

Comparisons were made to EUCAST[64] for their defined breakpoints. Breakpoints are the limits which define susceptibility and resistance, section 2.3.4.1.

5.2 MATERIALS AND METHODOLOGY

5.2.1 Preparation of inoculum

Bacteria were grown in the overnight culture then diluted to an O.D. reading of 0.01 [69]. Cultures were diluted by taking O.D. readings, and then used the following equation to calculate the required volume of the overnight culture which was needed to be added to fresh sterile broth:

$$OD1V1 = \quad OD2V2$$

Where:

OD1 = Initial OD reading of the overnight culture

V1 =? The unknown volume

OD2 = Desired OD reading, which in this case is 0.01.

V2 = 10 ml

Once V1 has been calculated, this volume of the overnight culture can be added to the fresh broth, check reading equals an O.D. of 0.01. The adjusted inoculum is equal to approximately 10^7 CFU/ml (based on O.D. v CFU/ml calibration curve not shown).

5.2.2 MIC microbroth assay

To determine the MIC for each of the antibiotics used against *A. baumannii* 96 well microtitre plates were prepared in advance by following a standard laboratory MIC microdilution assay protocol[71]. Final concentrations of antibiotics ranged from 0.5 - 512 mg/L for erythromycin, rifampicin, ampicillin, chloramphenicol and ciprofloxacin MICs and 256 – 0.25 mg/L for colistin. Plates containing antibiotics were stored at -20^{o}C for maximum of a week before use

and thoroughly defrosted before use; each plate contained 3 replicates and negative antibiotic controls. 100 µl of the adjusted inoculum (described in section 4.1.1) was added to the pre-prepared antibiotics and sealed using parafilm. Plates were incubated statically at 37^{o}C for 24 hours.

5.2.3 Minimum Bactericidal Concentration (MBC)

The MBC is the concentration at which an antibiotic is able to complete eradicate the bacteria. To do this, 10 µl spots of 3 wells either side of the first well to show no turbidity were plated on Müller Hinton agar plates and incubated for 24 hours at 37^{o}C; the lowest concentration where there was no growth was determined as the MBC.

5.2.4 Checkerboard MIC Assays

Antibiotic synergy was determined *in vitro* using the well-established microdilution broth checkerboard assay technique[70, 72]. The broad spectrum antibiotics (working concentrations ranging from 0.125 mg/L – 64 mg/L or 0.5 mg/L to 256 mg/L depending on initial MIC) were serially diluted across the ordinate whilst colistin (working concentrations 0.06 mg/L – 4 mg/L) was diluted along the abscissa. 100 µl of the adjusted inoculum was added and each plate was sealed and incubated statically at 37^{o}C for 24 hours.

5.3 RESULTS AND DISCUSSION

5.3.1 MIC

EUCAST[64] publish data on clinical MIC breakpoints (Table 4.2) provide investigators and clinicians information on the susceptibility of bacterial strains to different antimicrobials, see section 2.3.4.1.

Antibiotic	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Notes
	S ≤	R >		S ≤	R <	
Ampicillin	-	-		-	-	Susceptibility testing of *Acinetobacter* spp. to penicillins is unreliable. In most instances *Acinetobacter* spp. are resistant to penicillins.
Ciprofloxacin	1	1	5	21	21	
Erythromycin	-	-		-	-	
Tetracycline	-	-		-	-	
Chloramphenicol	-	-		-	-	
Colistin	2	2		See note	See note	Use an MIC method
Rifampicin	-	-		-	-	

Table 5.1: EUCAST Clinical Break points for *Acinetobacter spp.* '-' means no breakpoint information, susceptibility testing is not recommended. Table has been reproduced from EUCAST[64].

Antibiotic	MIC µg/ml	MBC µg/ml
Ciprofloxacin	> 16	16
Erythromycin	> 32	64
Chloramphenicol	> 32	32
Kanamycin	> 8	16
Tetracycline	> 1	1
Colistin	> 0.25	1
Rifampicin	> 2	2
Ampicillin	>512	512
Gentamicin	> 32	32

Table 5.2: MIC values of a range of antibiotics tested against *A. baumannii* 19606.

Comparing the values from the microbroth assay performed in this study to the clinical breakpoints as determined by EUCAST, it was determined that *A. baumannii* ATCC 19606 was susceptible to colistin only. The strain was determined as resistant to ciprofloxacin. For all the other antimicrobial agents tested in this study it is impossible to determine whether *A. baumannii* is susceptible or resistant as there are no break point values for these agents; however the values for all antibiotics, excluding rifampicin and tetracycline, were very high.

Although EUCAST recommend agents without break points should not be tested, in this study the MIC values for these agents needed to be determined in order to perform a checkerboard MIC assay. The synergist activity of ampicillin will not be tested due to the high MIC value recorded here.

5.3.2 Comparison to International antimicrobial wild type MIC distributions of microorganisms

International MIC distributions are graphical representations of different MIC ranges collated together from all kinds of different sources across the world. The MIC values have been obtained through a variety of different methodologies and although these different methodologies do not always yield the exact same MIC result, values rarely vary by more than one doubling dilution step[70]. By combining these MIC values into one graph, the distribution graphs are able to address the variation between different investigators and methodologies. The benefits of this mean that the information can be used as reference material for determining clinical breakpoints, epidemiological cut-off values, MIC ranges of wild type organisms. In addition, the MIC distributions also serve as an internal reference for calibration of antimicrobial susceptibility testing methods[70].

MIC distributions have many benefits, however they have also been heavily criticised due to their limitations. Data used in these has been collated from a wide variety of sources, time periods and some include high proportions of resistant organisms therefore may not be entirely applicable. Also many different studies use varying MIC ranges making a comparison using these results difficult, especially when comparing the upper part of the MIC ranges as they may not exist.

In terms of the current study the international MIC distributions were used as an internal standard. For antimicrobials colistin, ciprofloxacin, rifampicin and gentamicin distribution data was available, Figure 5.1. The MIC values fall within the MIC distribution values reported by EUCAST. Data was not available on the EUCAST for the MIC distributions of erythromycin, chloramphenicol, kanamycin and ampicillin against *A. baumannii* wild-types organisms.

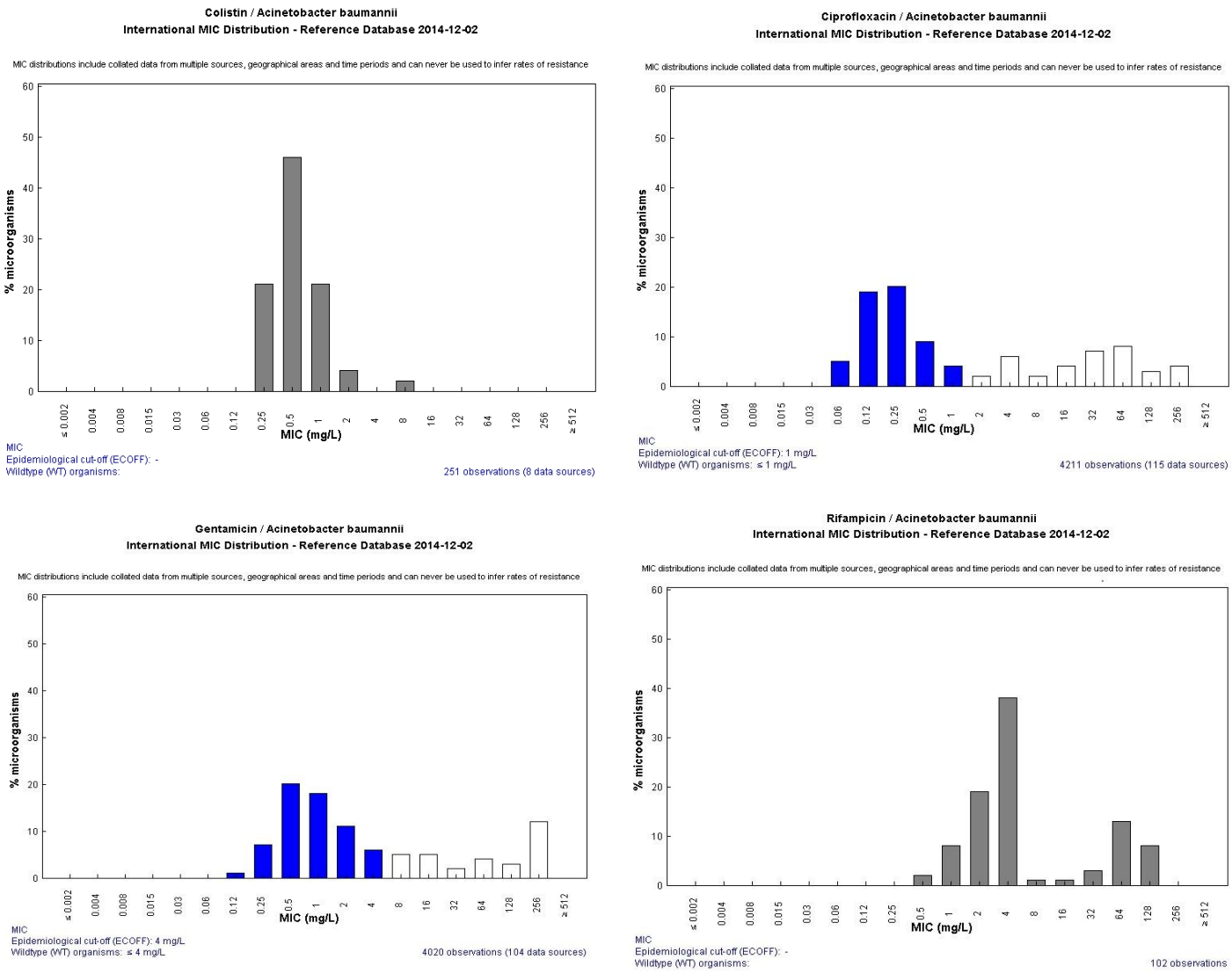


Figure 5.1 MIC distributions for a) colistin b) ciprofloxacin c) gentamicin d) rifampicin against pathogenic *A. baumannii* collected from multiple sources, geographical areas and time periods (Solid lines = wild type). EUCAST 2014[64].

5.3.3 Checkerboard Minimum Inhibitory Concentration Assay

Degree of synergy between antimicrobial drugs was expressed in terms of the Fractional Inhibitory Concentration (FIC), which can be calculated using the following equation:

$$\text{FIC} = \text{MIC of drug in combination} / \text{MIC of drug alone}$$

From the calculated FIC values the fractional inhibitory concentration index (FICI) was defined as the lowest FIC value. Antibiotic concentrations were defined as synergistic if the FICI ≤ 0.5, indifferent if the FICI between 0.5 and 4 or antagonistic if the FICI >4.0[72]. If combinations are determined as synergistic, then the effect of the two antibiotics used together is greater than if the agents were to be used alone.

Antibiotic	FICI	Synergistic/Indifferent/Antagonist
Ciprofloxacin	0.63	Indifferent
Erythromycin	0.37	Synergistic
Kanamycin	0.75	Indifferent
Tetracycline	0.75	Indifferent
GentamIcin	0.75	Indifferent
Chloramphenicol	0.63	Indifferent
Rifampicin	0.13	Synergistic

Table 5.3: Determination of synergy/indifference/antagonism for various broad spectrum antibiotics in combination with colistin.

Erythromycin alone displayed little activity against of *A. baumannii* 19606 MIC >32 mg/L, table 5.2, and due to the insufficient information on the clinical breakpoints for erythromycin against *A. baumannii* it was impossible to determine whether *A. baumannii* was resistant or susceptible to erythromycin, table 5.1. It is for this reason, EUCAST[64] recommended that erythromycin should not be used as a treatment option for such infections as the therapeutic outcome is unknown and likely poor. However the combination of erythromycin and colistin resulted in an FICI of 0.37 mg/L, which means the combination is synergist, table 5.3. Therefore it is possible to conclude from this that when used in combination with each other the two antibiotics produce an effect that is greater than if they were to be used alone. What

this means in the present study is that when the combination of the antibiotics is administered to individuals in models deliberately infected with *A. baumannii* it is expected that a greater number of individuals will survive the infection.

For rifampicin, again the insufficient information available on the clinical breakpoints for rifampicin against *A. baumannii* it was impossible to determine whether *A. baumannii* was resistant or susceptible to rifampicin. EUCAST in their clinical breakpoint table recommend that rifampicin should not be used in susceptibility testing against *A. baumannii* infections, however the MIC value from this study suggest a high level of activity, which suggests activity against *A. baumannii* ATCC 19606. When used in combination with colistin, the FICI was determined as 0.13 (table 4.8) the lowest calculated FICI of all the antibiotic combinations tested. As the FICI is below 0.5, the combination is defined as synergistic, producing an effect combined that is greater than if either antibiotic was to be used alone.

Due to the insufficient information on the clinical breakpoints for kanamycin against *A. baumannii* it was impossible to determine whether *A. baumannii* was resistant or susceptible to kanamycin, table 5.1. It is for this reason, EUCAST[64] recommended that erythromycin should not be used as a treatment option for such infections as the therapeutic outcome is unknown and likely poor. In combination with colistin the FICI was determined as 0.75 (table 5.3), an FICI value greater than 0.5 but lower than 4 therefore the combination has been defined as indifferent, therefore therapeutic success is no greater than if the agents were to be used alone. Tetracycline, chloramphenicol, ciprofloxacin and gentamicin also all had FICI values calculated which fell within the indifferent range and therefore the combination with

can be determined as indifferent, indicating there would not be any advantage to combining the two antibiotics for the treatment of MDR *A. baumannii*[72].

5.3.4 Limitations

Due to the insufficient information on the clinical breakpoints for many broad spectrum antibiotics against *A. baumannii* this makes it incredibly hard for investigators and clinicians to research/make decisions when I comes to approaching the MDR problems surrounding *A. baumannii*. To add to the lack of information available is an interesting point that even when the major organisations that define the breakpoints (CLSI and EUCAST), these have been shown to be different for many of the key antibiotics used in the therapy of *A. baumannii* infections[5]. This conflicting information and the fact that EUCAST report insufficient evidence for the majority of antibiotics leads to much confusion surrounding the treatment options available for infections caused by MDR *A. baumannii*[64]. The impact this has on research is a lack of understanding of appropriate dosing levels and agents. In a recent conference meeting hosted by ESCMID on reviving old antibiotics, it was concluded that the limiting knowledge of the behaviour of old antibiotics and the resistant mechanisms of MDR bacteria has led to the misuse and mistreatment of MDR infections[73]. MICs of antibiotics against such bacteria need to be reviewed to gain a better insight into possible successful therapeutic options.

Colistin is a cationic antibiotic which creates many problems throughout the preparation and testing of this antibiotic, due its affinity to bind to negatively charged plasticware. The binding of colistin to the walls of plastic Eppendorfs and 96 well plates means there is less 'free'

colistin available to act upon the bacteria. This could impact the concentrations originally prepared and also the activity of colistin *in vitro*. Unfortunately it is also the cationic characteristic of colistin which prevents antibiotic disk susceptibility being carried out. The cationic charge of the colistin from diffusing through Müller Hinton agar; these issues have called for EUCAST to formulate a method in which the MIC testing of colistin is standardised. In a recent conference meeting hosted by ESCMID[73], issues surrounding the conflicting results being published involving colistin antibiotic testing was raised and it was concluded more work needed to be done to standardise the testing and for the evaluation of antimicrobials against MDR bacteria.

The checkerboard MIC assay is widely used for the evaluation of *in vitro* synergy for multiple agents, however problems with the performance, standardisation and interpretation have been discussed[74]. One major limitation discussed is the inherent error of the two-fold dilution of the antibiotics being assessed. The difference between each concentration is half of its predecessor, which leads to the precision of this technique being questionable. However although these limitations are concerning, it is important to bear in mind that the principle of examining the growth of bacteria at multiple dilutions of combined antibiotics *in vitro*, is valid for the assessment of synergy but may be further developments need to be undertaken to improve this[74].

5.4 SUMMARY OF RESULTS

Of all the antibiotics, only rifampicin and erythromycin where calculated to be synergistic when in combination with colistin. All other antibiotic combinations with colistin were found to be indifferent, meaning the activity of the antibiotic in combination with colistin did not inhibit or improve the activity of the drug against *A. baumannii*. None of the combinations were found to be antagonists. However although this suggests that the combinations will have little improvement when evaluated *in vivo*, previously combinations assessed *in vivo* that *in vitro* had shown to be indifferent, significantly improved the survival rates[13].

6 *IN VIVO* EVALUATION OF ANTIMICROBIAL COMBINATIONS

6.1 INTRODUCTION

Based on the MIC/FICI values from the previous section, it was possible to determine that erythromycin and rifampicin are most likely to have a synergist effect when combined with colistin in treating *A. baumannii* infections. To evaluate this *in vivo*, the *G. mellonella* model was employed.

6.2 METHODS AND MATERIALS

6.2.1 Mono-therapy

To investigate the efficacy of colistin, erythromycin and rifampicin against *A. baumannii* infections, groups of 16 larvae were challenged with lethal doses of *A. baumannii* then treated with a single dose of antibiotic, see table 5.1 for dosage. Doses used in this experiment were based on human doses previously described in the literature, see table 6.1. Control pathogenicity and PBS groups were included in each repeat, experiments were repeated three times. Immediately after each treatment, the larvae were incubated at 37^{o}C and their survival rate recorded every 24 hours over 96 hour period.

Antibiotic	Dose
Colistin	2.5 mg/kg[15]
Erythromycin	50 mg/kg[75]
Rifampicin	8 mg/kg[76]
Gentamicin	10 mg/kg[13]
Chloramphenicol	12.5 mg/kg[77]
Ciprofloxacin	20 mg/kg[18]
Ampicillin	20 mg/kg[22]
Kanamycin	7.5 mg/kg[78]

Table 6. 1: Dose calculated based on current literature for hospital acquired infections.

6.2.2 Combination therapy

To investigate the efficacy of the antibiotic combinations, larvae groups were injected at the doses described above and injected with antibiotics in combination. To allow for the trauma associated with double injections, uninfected larvae inoculated twice with 10 µl of PBS were also used. Immediately after each treatment, the larvae were incubated at 37^{o}C and their survival rate recorded every 24 hours over 96 hour period.

Control pathogenicity and PBS injected larvae were included in each repeat for all *G. mellonella* experiments. Groups where 2 or more larvae from the control groups died were rejected.

6.3 RESULTS AND DISCUSSION

As suggested in the *in vitro* analysis of ciprofloxacin, gentamicin, ampicillin, kanamycin and chloramphenicol in combination with colistin, these combinations were unsuccessful in improving the survival rate of *G. mellonella* groups that had been experimentally infected

with pathogenic levels of *A. baumannii.* All groups treated with these antibiotic combinations gave a very poor survival rate ≤6.25% and due to this, results have not been shown.

However, the combination of both erythromycin and rifampicin with colistin was significantly more active than either drug alone in the infected *G. mellonella* model, table 6.2. Rifampicin combinations resulted in the highest percentage survival and therefore most active combination against *A. baumannii* infected groups.

Antibiotic	% *G. mellonella* survival after 96 hours	
	Treatment alone	Treatment in combination with colistin
Erythromycin	4.17±3.61	35.42±7.22
Rifampicin	12.5±6.25	43.75±3.61
Colistin	14.58±3.61	-

Table 6.2: antimicrobial treatment efficacy against planktonic *A. baumannii* ATCC 19606 infections evaluated in a *G. mellonella* infection model

Larval groups infected with lethal planktonic cultures of *A baumannii* ATCC 19606, following treatment with colistin alone, the survival rate was only 14.58% (±3.61 sdp). Treatment with colistin in combination with rifampicin (43.75% ± 3.61 sdp) or erythromycin (35.42% ± 7.22 sdp) showed combination therapy was significantly more active in treating ATCC 19606 lethal infections (t test, P = < 0.01 for both).

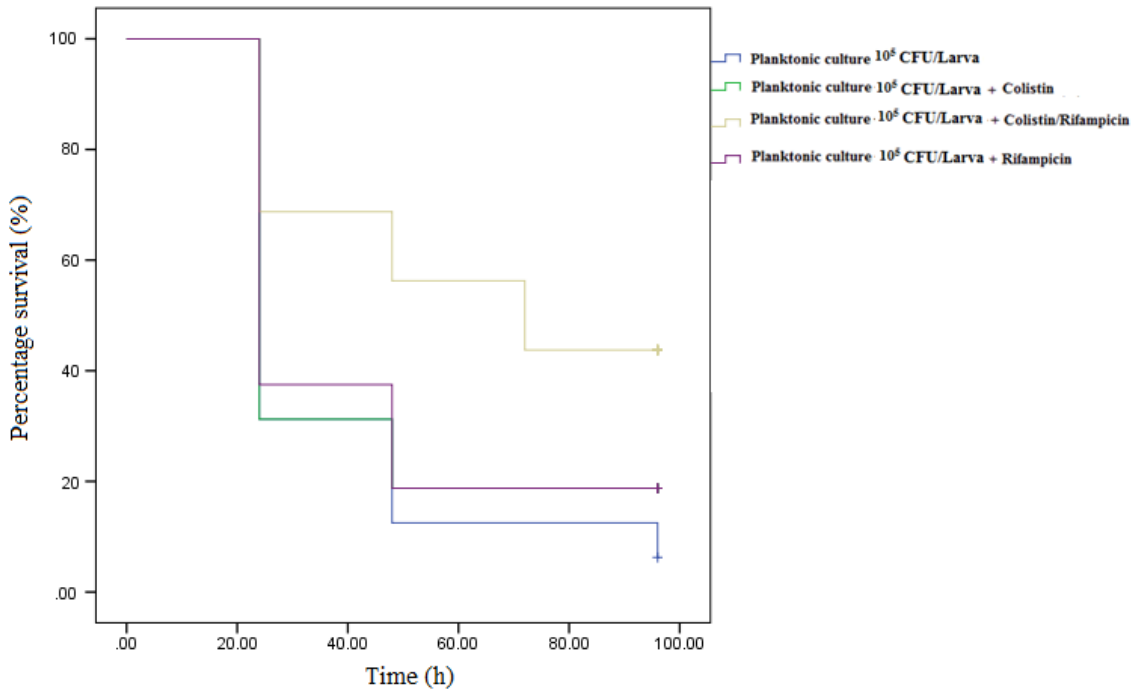


Figure 6.1: Kaplan Meier survival curve comparing the treatment of lethal *A. baumannii* ATCC 19606 infections with rifampicin and colistin alone and in combination in the *G. mellonella* infection model. Survival was monitored every 24 hours over 96 hour period. Graph represents results from a single typical test.

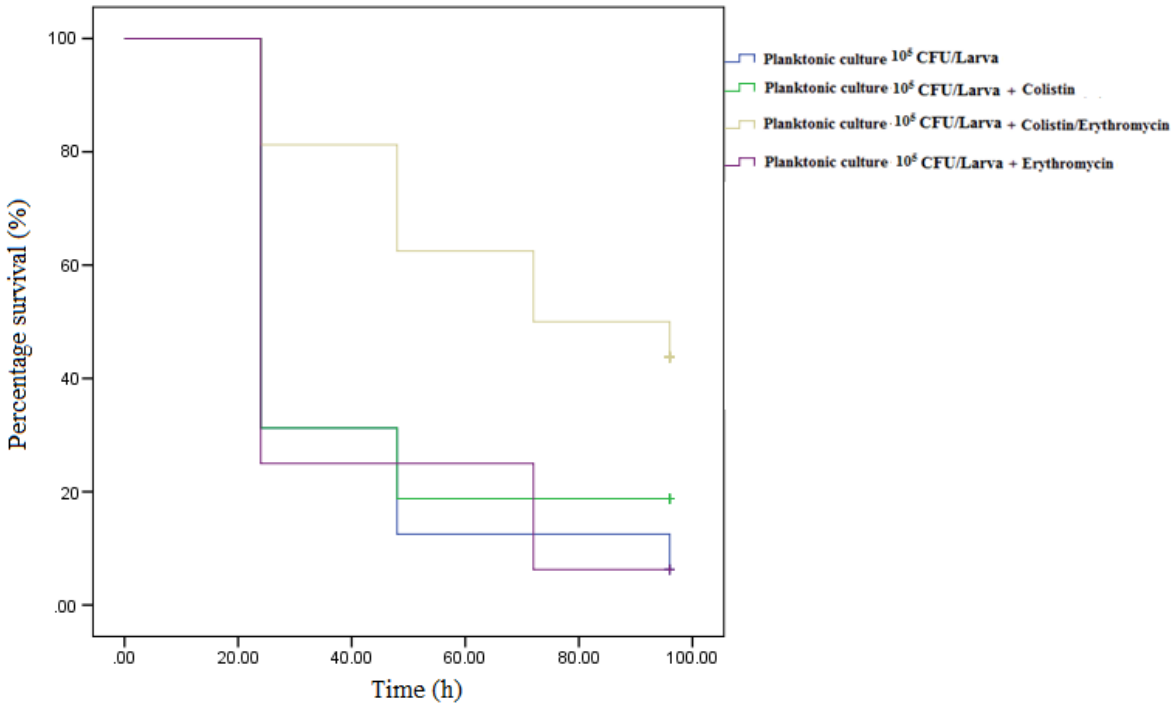


Figure 6.2: Kaplan Meier survival curve comparing the treatment of lethal *A. baumannii* ATCC 19606 infections with erythromycin and colistin alone and in combination in the *G. mellonella* infection model. Survival was monitored every 24 hours over 96 hour period. Graph represents results from a single typical test.

Both graphs show typical infection groups that have been treated with either erythromycin or rifampicin in combination with colistin. Both treatment combinations were found to significantly improve the survival rate of the infected larval groups $P<0.05$.

It has been proposed that the synergistic effects observed with colistin/ polymyxin B are due to the disruption of the outer membrane permeability. Although this interpretation is reasonable, the dual action of polymyxin antibiotics introduces a degree of uncertainty.

Once the outer membrane has been disrupted by colistin (a non-lethal action), free colistin are able penetrate the inner layers of the cell envelope resulting in leakage of the cytoplasmic

contents, which is lethal[13, 15, 31]. Both actions can occur at the same polymyxin concentration, however studies have found that it is possible to separate the two actions with the outer membrane permeabilisation activity occurring at a lower dosage.

Previous studies report that colistin when tested *in vitro* initially displays bactericidal activity against clinical strains of *A.baumannii* but this appears to be overcome after 24 hours[15]. The present study agrees that colistin does not possess sufficient bactericidal activity to overcome initial *A. baumannii* infections in the first phase of treatment. Since colistin demonstrates excellent synergy *in vitro* with both erythromycin and rifampicin, and larval survival is significantly increased in the combination model of infection, suggesting there is sufficient bactericidal activity within the combinations that is able to overcome the infection. Cai *et al.*[16] analysed antimicrobial strategies to avoid colistin resistance of *A. baumannii* comparing a number of patient studies evaluating combination therapies of colistin with a range of antibiotics, including rifampicin. Rifampicin/colistin combinations were shown *in vitro, in vivo* and in clinical reports to be promising therapeutic options against MDR *A. baumannii* infections. The model evaluated in the present study supports these finding and also provides evidence on the use of the rifampicin/colistin combination therapy against *A. baumannii* infections in a simple, cheap and ethically acceptable model.

Other macrolide antibiotics in combination with colistin have been shown previously to be synergists *in vitro*; Timurkaynak *et al.* [79] found azithromycin in combination with colistin to be synergistic against three different *A. baumannii* isolates. In the present study, the macrolide erythromycin was found to both be synergist *in vitro* and significantly improved survival of the *G. mellonella.*

6.4 SUMMARY OF RESULTS

When assessed *in vivo* not all of the colistin combinations were found to be effective and significantly improve the survival of the larva. It was only the combination of erythromycin and rifampicin with colistin which was calculated to be significantly more active than not only the drugs alone but also the other combinations in the infected *G. mellonella* model. Rifampicin combinations resulted in the highest percentage survival overall and therefore most active combination against the infected groups.

7 BIOFILM FORMATION, PATHOGENICITY AND TREATMENT

7.1 INTRODUCTION

Bacteria have evolved to become proficient at adapting to not only their extracellular surroundings but also environmental conditions, which has made it possible for them to attach and form biofilms in nearly all habitats where life can exist. This has resulted in major health concerns and economic burden in both hospital and industrial environments[37, 80-87]. According to reports by a team of researchers at the Centre for biofilm Research in Montana, it was not until the 1970s that scientists began to appreciate that bacteria predominately exists as biofilms. Before this, bacteria was perceived as a unicellular life form existing in a pure-culture paradigm for the results to be experimentally valid, when in fact biofilm associated microbes are very different from their planktonic counterpart on a surface, displaying an altered phenotype in terms of growth rates and gene transcription, when compared to planktonic cells of the same organism[36, 88]. This has resulted in an inherent error in that this is not a true representation of how the majority of bacteria exist outside the laboratory environment[42]. As previously discussed in section 2, biofilms do not exist as pure culture single species, rather a multispecies communities encased within the biofilm matrix. In the present study the biofilm-associated suture model was preliminarily used to develop a novel method with which biofilm associated infections could be studied and also to assess whether a mixed species biofilm would have an effect on the resistance of *A. baumannii.* The organisms which will be studied alongside nosocomial important strain *A. baumannii* ATCC 19606 is *E. coli* ATCC 25922.

E. coli ATCC 25922 is an ideal strain to be used in laboratory investigations as it has been reported as a suitable surrogate for the human pathogenic strain *E. coli* O157:H7, a common cause of food poisoning, due to the similar biofilm forming capabilities[37, 89, 90].

7.2 Materials and Methods

7.2.1 Biofilm model

To study the biofilm infection within the *G. mellonella* model a suitable material needed to be selected to which the bacteria could adhere to and also easily injected in to the model. Mammalian infection models use to investigate biofilm infections associated with indwelling devices use actual devices to simulate the infections *in vivo* [86]. Due to the size of the *G. mellonella* larvae, it is not a viable option to use such simulations. Therefore an alternative material was required. Based on the traditional staphylococcal string infection model in rodents, where 'string' contaminated with bacteria was sewn under the skin of mammalian, a method was formulated in which bacteria would be grown on to the 'string' and manipulated into the *G. mellonella* model.

Varying lengths of the suture was initially cut to determine a manageable length of suture which would enable it to insert into the larva; lengths ranged from 1 to 3 mm. Ultimately it was decided that the suture would be cut into 3mm pieces as this allowed for easy handling and accurate cutting.

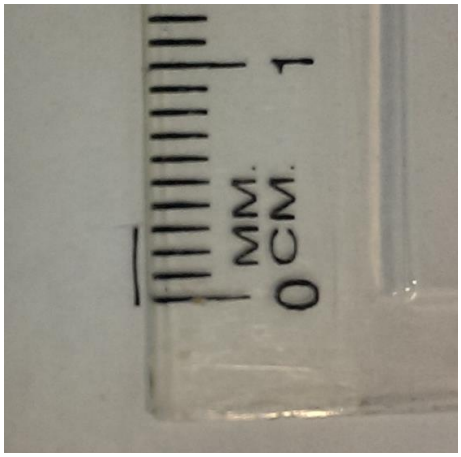


Figure 6.1: scaled photo of cut surgical suture measuring 3 mm.

7.2.2 Insertion trauma

Previous preliminary work carried out by the established whether there was any difference between injecting using a pipette and sharpened pipette tips (Eppendorf, US) verses using a Hamilton 10 µl syringe needle (Sigma-Aldrich, UK). To develop a method for inserting the suture pieces in to the *G. mellonella,* individual sterile pieces of suture were aseptically inserted in to the bottom of sterile p200 pipette tips (Sarsteadt, Germany) which had been sharpened to an angle that mimicked the needle of the SGE 10µl syringe needle.

In order to insert the suture in to individual larvae, the sharpened pipette tip penetrated the left proleg, then with the aim of a sterile stainless steel wire the suture piece was very gently inserted in to the body of the larvae. A sample size of 16 larvae was used in each test situation, and control included groups which received no manipulation and groups which mimicked the trauma of the suture being inserted. All groups were incubated at 37^{o}C and the survival rates were monitored for 96 hours and the results statistically analysed using Kaplan Meier survival analysis.

7.2.3 Establishing growth on suture surface

Once it had been establish that the larvae could survive the trauma of being injected with sterile suture via a pipette tip, the growth of each species both individually and as a mixed culture needed to be established upon the individual suture pieces. To do this for the single species attachment, an overnight culture of each species was first adjusted to a culture with an O.D. of 0.01, see section 5.2.1. 2 ml of the adjusted inoculum was added to 5ml bijous containing approximately 20 autoclaved suture pieces and incubated at 37^{o}C with RPM of 125 for 24 hours. Individual suture pieces were then removed aseptically and washed thoroughly with PBS. For the mixed species attachment, the individual inoculums were adjusted to cultures with O.D. values of 0.01, and at a ratio of 0.42:0.29 as previously defined (P.Skipper personal communication at the University of Lincoln) 100 ml of mixed species inoculum was produced 2 ml of the mixed species inoculum was then aliquoted in to the 5ml bijoux containing the autoclaved sutures and incubated as described above.

7.2.4 Visualisation of biofilms

7.2.4.1 Enumeration of bacteria in biofilm growth phase

In order to distinguish between the two species when plated out on to agar, a selective agar Membrane Lauryl Sulphate Agar or MLSA was used. MLSA is usually used to detect coliform organisms and *E. coli* in water. The lactose-fermenting colonies appear yellow on the agar plates and can be used as a presumptive test for *E.coli. A. baumannii* appears as pink colonies on the agar surface as these organisms are non-lactose fermenting (NLF) organisms. To test for the presence of NLF after 24 hours of growth, individual pieces of suture were aseptically removed for the bijoux and placed in a new sterile 1.5ml Eppendorf containing 1ml of saline. The Eppendorf was then vortexed for 5 minutes to disrupt the biofilm from being attached to

the suture surface. Eight serial dilutions of this suspension were prepared and spread plated on to the MLSA plates and incubated at 37^{o}C overnight. The morphology and colour of the colonies was recorded.

In order to count of bacteria attached to individual suture pieces, the same procedure as above was performed, and the number of colonies was also recorded and the plates were read using a Scan® 500 plate reader (Interscience, France).

7.2.4.2 Gram staining and Scanning Electron Microscope

To assess the extent of biofilm growth on the suture, the suture was visualized using a high powered light microscope, individual suture pieces were aseptically removed from the bijou they had been incubated in using sterile forceps. Each piece was then rinsed twice using sterile PBS and gently placed on a clean glass slide. The ends of the suture were then carefully taped down to glass slides. The glass slides were then fixed and stained following Gram's staining protocol and visualised under a high powered light microscope at a total magnification of x1000[91].

Suture pieces with *A. baumannii* attached to them were also visualised using scanning electron microscope. SEM is a powerful technique which enables the surface visualisation of samples at high magnifications. The electron microscope produces images by using a focused electron beam to generate a variety of signals that derive from electron-sample interactions, which reveal information about the sample including surface morphology. In this study the SEM was used to visualise both planktonic and biofilm cultures of *A. baumannii*.

To visualise biofilm cultures, samples were grown on the suture as described in section 7.2.3. These samples were washed twice with PBS, and prepared for SEM examination by washing with 0.1M sodium cacodylate and fixing in 2.5% glutaraldehyde in 0.1M sodium cacodylate

for 30 minutes. Specimens were then wash twice in dH_2O, dehydrated for 10 minutes at each stage of an ascending ethanol series (50% to 100%) and left to air dry[92]. Each sample was sputter coated in Carbon before visualization in the SEM. Samples were analysed under a FEI scanning electron microscope at a working distance of 10 mm (Quanta SEM, FEI™, USA).

For the planktonic culture specimens, 1ml of an overnight culture was transferred to a new sterile Eppendorf and centrifuged at 11,000 xg for 3 minutes to form a pellet. The fixing procedure then followed the same procedure as for the biofilm samples.

If at any point the pellet dissolved into the solutions, the mixture was centrifuged to form a pellet once more.

7.2.5 Pathogenicity and treatment of biofilm attached to suture

7.2.5.1 Checkerboard Assay

As determined in Sections 4 and 5, the *in vitro* activity of colistin in combination with rifampicin or erythromycin was significantly better than that of the other broad spectrum agents in combination with colistin. Therefore the same combinations were used in the analysis of the *in vitro* activity of biofilm mode *A. baumannii*.

Antibiotic synergy was determined *in vitro* using the well-established microdilution broth checkerboard assay technique [68]. The broad spectrum antibiotics (working concentrations ranging from 0.125 mg/L – 64 mg/L or 0.5 mg/L to 256 mg/L depending on initial MIC) were serially diluted across the ordinate whilst colistin (working concentrations 0.06 mg/L – 4 mg/L) was diluted along the abscissa. Plates were stored in a -20^{o}C freezer then thoroughly defrosted before using. To assess the activity of bacteria and subsequent biofilm formation, 100 µl of adjusted inoculum was aseptically added to flat bottomed 96 well tissue culture

plates. The peg systems were then carefully placed on top, each plate was sealed and incubated statically at 37°C for 24 hours. After 24 hours, the MBEC™ peg lids were then carefully and aseptically removed from the inoculated plate, rinse twice with PBS and added to the antibiotic checkerboard plates, which were then sealed and incubated at 37°C for 24 hours.

7.2.5.2 In vivo pathogenicity and therapeutics

7.2.5.2.1 Single species

Before the pathogenicity of the biofilm-associated infection could be assessed, it was essential to establish the pathogenicity of *E. coli* ATCC 25922 as a planktonic culture, in order to understand the behaviour of the bacteria and to be able to make a comparison. The pathogenicity of ATCC 25922 was established by following the same methodology described in section 3.2.3.

To assess the pathogenicity of both single and mixed species biofilm-associated infection, 25922 biofilms were grown on 3mm lengths of suture as previously described above in section 7.2.3. After washing twice with PBS, each suture was carefully transferred to a sharpened sterile 20µl Gilson-type pipette tip (Sarstedt, Germany). The pipette tip was gently inserted in to the top left proleg of larvae and a fine needle was used to insert the biofilm suture into the body of the larvae. The groups of larvae were then incubated at 37°C for 96 hours alongside controls and survival rate was recorded every 24 hours. The degree of melanisation of each larva was also recorded. Larvae were determined as dead if they failed to respond to touch after 1 minute of stimulation.

After the suture had been inserted, groups which were to receive either mono- or combination antibiotic treatment did so in the manner previously described in section 5.2.

7.3 RESULTS AND DISCUSSION

7.3.1 Insertion trauma

Immediately after stimulating the trauma associated with inserting the pipette tip into the proleg of individual initial observations of the different injection methods showed great deal of trauma was inflicted on the larval groups with a loss of hemolymph, however it was noted within minutes, a black scab like plug had formed around the entrance of the proleg (where the tip had entered) and the larvae continued to behave in their usual manner and so the survival rates of the two groups where then monitored over 96 hours to see if the trauma had any adverse effects on the survival rate of the tested group.

Figure 7.1 shows the results of the survival analysis of the different trauma groups carried out over a 96 hour period. The graph shows after 96 hours, there was 100% survival rates in both the negative control and the group experiencing the trauma associated with the pipette injection. The Chi-squared statistical value for the difference between these groups was 4.131 with an associated P-value of less than 0.127 (95% confidence level) which is greater than the accepted level of statistical significance employed in this work is ($P < 0.05$) therefore it is possible to conclude that the trauma experienced in inserting the sterile suture via a sharpened pipette tip, does not cause a significant difference in survival rates compared to groups that receive the trauma of the tip alone or no trauma at all.

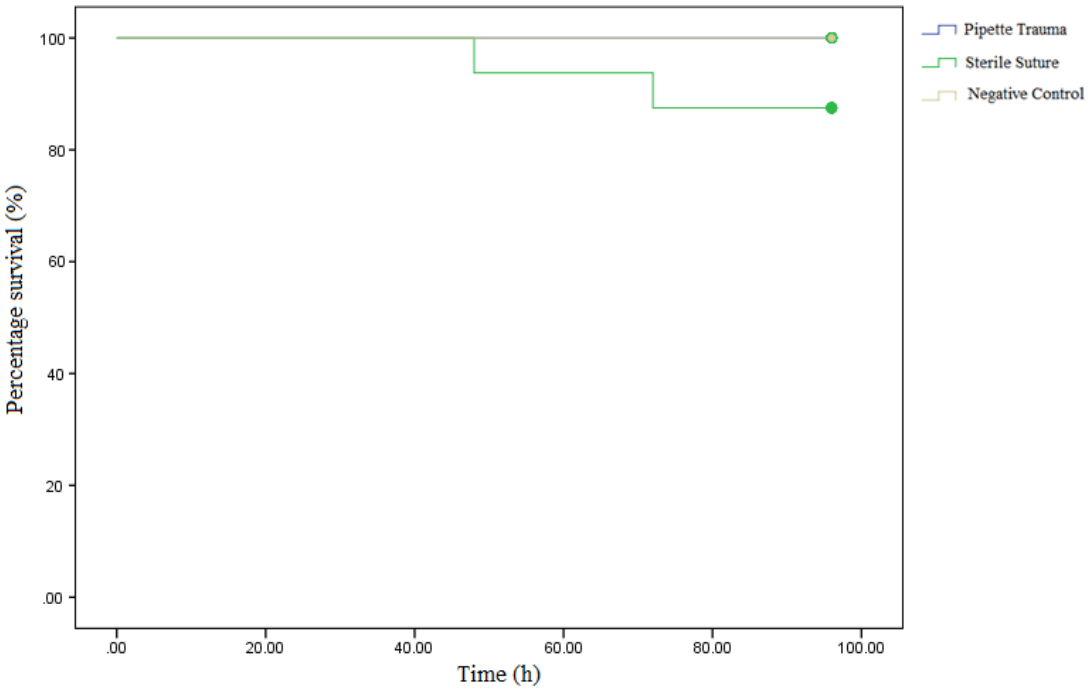


Figure 7.1: Kaplan Meier survival curve comparing the trauma experienced through inserting the suture in to individual larvae over a 96 hour test period.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	4.131	2	.127

Table 7.1: The overall comparison for testing the equality of survival distributions for the different trauma groups.

As mentioned briefly before, wound healing capabilities of *G. mellonella* larvae has been extensively studied by Rowley and Ratcliffe[63]. They found immediately after wounding both fat-body and hemolymph of the larvae were forced through the wound to form a plug. At the same time, the hemolymph coagulated forming vast networks of strand-like material attached to the hemocytes underlying the wound. After one to two hours this plug melanized

and the cells became highly necrotic. Six hours after wounding, there was a massive influx of hemocytes which eventually attached to the melanized layer over the wound to form a multicellular sheath. Twelve to twenty-four hours later, the epidermal cells underlying the broken cuticle detached and migrated across the wound to form a new intact layer. This intricate wound healing process, similar to that of the mammalian process, could explain why the trauma of the pipette tip was not found to significantly affect the survival rates of larvae.

An important benefit to bear in mind with this model of biofilm injection is the ability to inject the suture in this manner is that the whole system becomes disposable. This minimises the risk of contamination and reducing the work load between injections as a cleaning step between each injection does not need to be employed. Therefore reducing time and work load during the administration of the suture in the Galleria model.

7.3.2 Biofilm growth

7.3.2.1 Enumeration of attached bacteria

Initially, serial dilutions of vortexed suture in saline were plated on to MLSA plates in order to distinguish between species, Figure 7.1. The control plates clearly show the distinction between lactose-fermenting *E. coli* colonies (yellow colonies, figure 7.1a) and not lactose-fermenting *A. baumannii* colonies (pink colonies, figure 7.1b). Figure 7.1c shows a plate which belongs to a suture that had a mixed species biofilm grown on it, both yellow and pink colonies grew on this plate helping to confirming the mixed growth of both *E. coli* and *A. baumannii* on the suture.

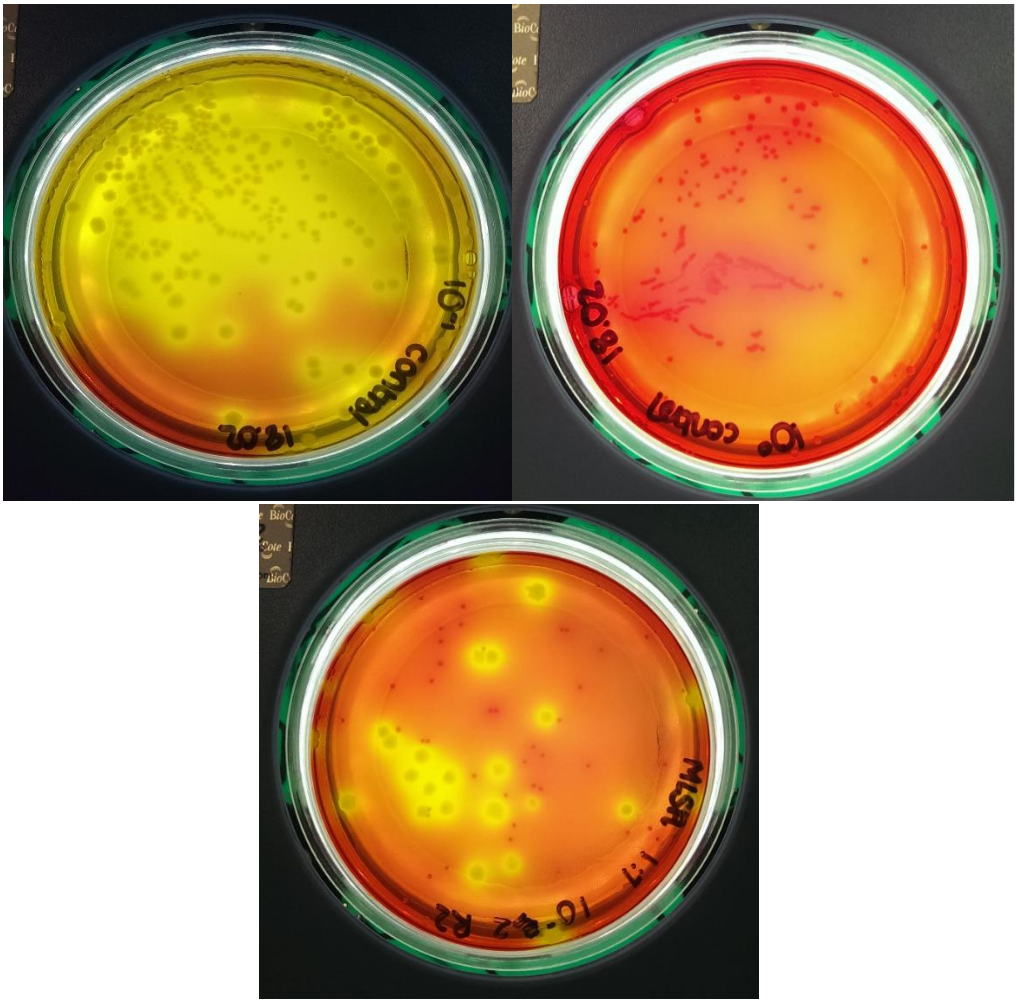


Figure 7.1: MLSA agar plates showing the distinctive differences between the two bacterial strain, *E. coli* ATCC 25922 and *A. baumannii* ATCC 19606. a) *E. coli* ATCC 25922 control plate b) *A. baumannii* ATCC 19606 control plate c) Mixed-species vortexed suture spread plate.

As before, for the enumerate viable bacterial colonies, an assumption is made that each bacterial colony on immobilised on the agar plate arose from one living (viable) cell, therefore it is possible to determine the number of live bacteria defined as colony forming units per ml

of the original culture, with the general acceptance range for counting colony forming units being 30 to 300 colonies. Table 7.1 shows the results of the vortexed suture

Dilution	CFU/ml
A. baumannii	1.37×10^6
E.coli	4.29×10^6
A.baumannii (mixed species suture)	3.69×10^8
E. coli (mixed species suture)	6.95×10^8

Table 7.2: Average CFU/ml values for *A. baumannii* ATCC 19606 and *E. coli* ATCC 25922 grown as single and mixed species biofilms on surgical suture.

7.3.2.2 Gram Staining and SEM analysis

The attachment of *A. baumannii* ATCC 19606 bacteria on the surgical suture pieces was investigated using Gram staining and SEM.

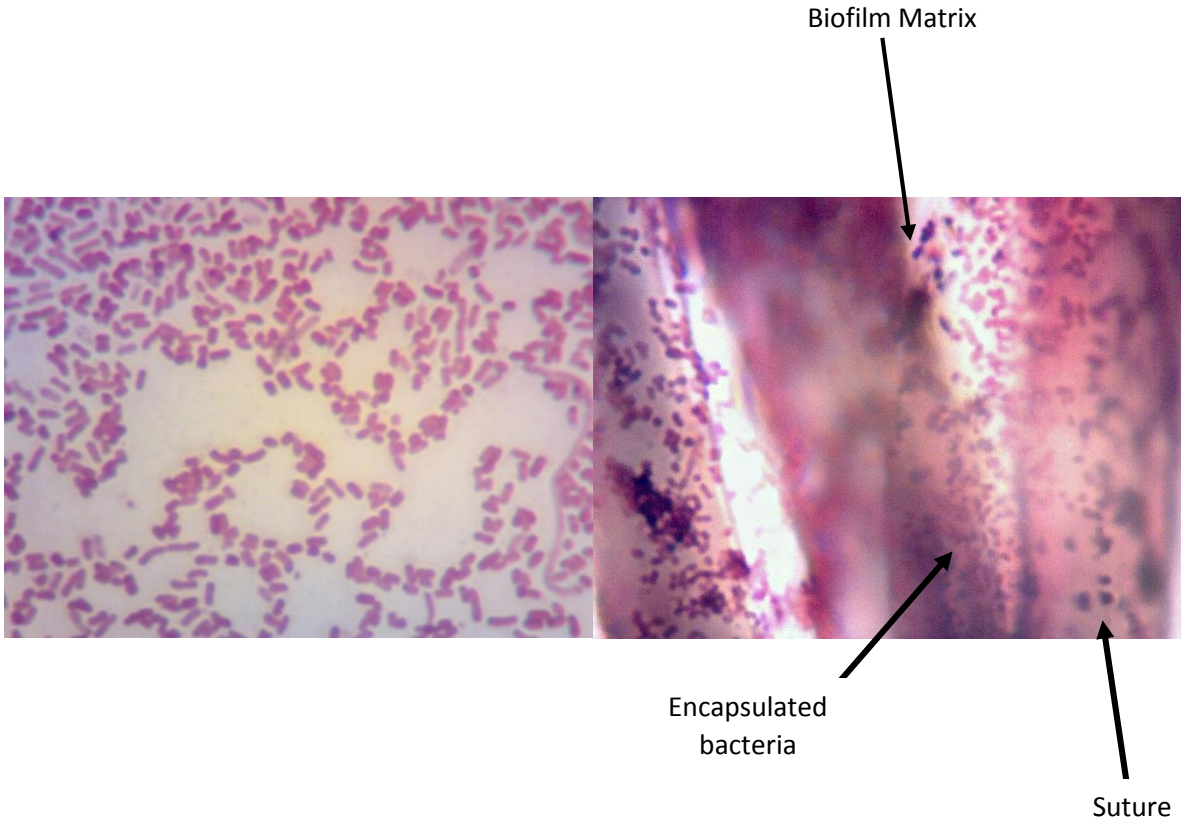


Figure 7.2: Gram stain section of *A. baumannii* ATCC 19606 biofilm grown on surgical suture. Helicon focus software was used to overlay images to produce a clear focused in image.

Gram stains distinguish between positive and negative bacteria by the dye that is retained within the bacterial cell walls. Gram positive bacteria hold on to the primary dye-iodine complex and stain violet. Gram negative bacteria are decolourised during the Gram stain process and take up the final dye stage, Safranin, and stain pink[91, 93]. The reasoning for the differences in staining is due to the properties of the bacteria's cell wall. Gram negative

bacteria have thinner layered cell wall and also a larger number of lipids present in the walls compared to the cell walls of Gram-positive bacteria which may explain the differences in the Gram stain reactions[93]. As *A. baumannii* is a Gram-negative, the bacteria Gram stain should be pink. However *A. baumannii* is notoriously known as a difficult species to Gram stain, and therefore this can lead to misidentification.

Encapsulated *A. baumannii* cells are clearly visible within the biofilm matrix in Figure 7.2 and the image shows the Gram negative coccobacillus shaped *A. baumannii* either attached directly to the suture, or within a translucent tent-like structure - the extracellular matrix formed from bacterial cell secreted extracellular polymeric substances.

The appearance of biofilm formed by *A. baumannii* ATCC 19606 was also investigated using SEM. As a control, bacteria of the same isolate in planktonic growth phase were immobilised and analysed with the SEM. SEM analysis of the biofilm grown on suture samples show attached *A. baumannii* cells to the surface of the suture across all visible surfaces. Figure 6.3(b) shows an SEM image at a higher magnification of bacteria adhered to the suture.

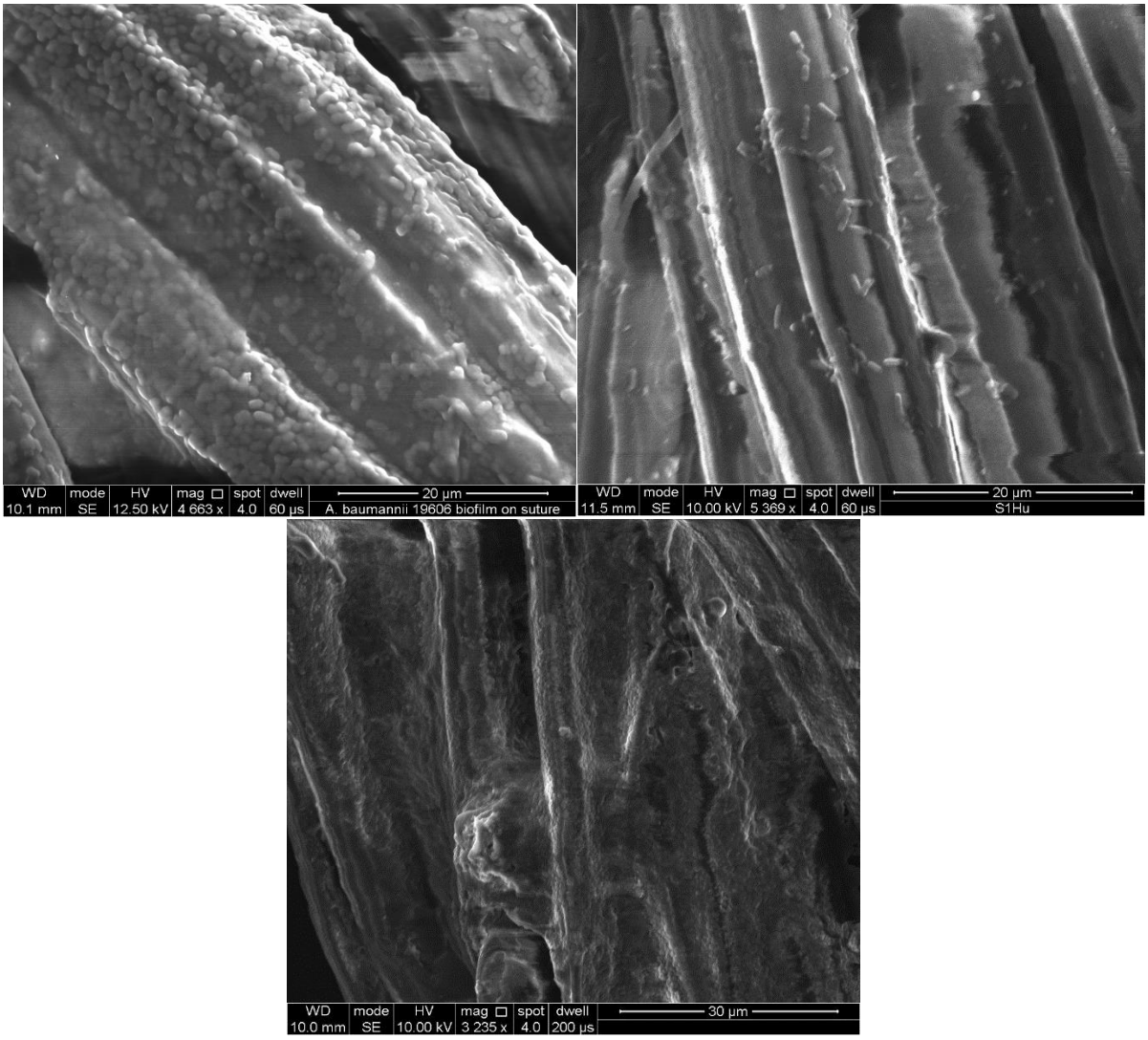


Figure 7.3 (a) Scanning electron image of biofilm growth phase *A. baumannii* ATCC 19606 adhered to suture surface. (b) Scanning electron image of biofilm growth phase *E.coli* ATCC 25922 adhered to the suture surface. (c) Scanning electron image of mixed species biofilm growth phase of *A. baumannii* ATCC 19606 and *E.coli* ATCC 25922 adhered to the suture surface.

SEM analysis of the biofilm grown on suture samples show attached *A. baumannii* cells to the surface of the suture across all visible surfaces. Figure 7.3(b) shows an SEM image at a higher magnification of bacteria adhered to the suture.

Sparse EPS matrix surrounding bacterial cells of the single species biofilms, however the SEM image of the mixed species biofilms showed the EPS matrix clearly distinguishable on covering the micro-colonies. The combination of the two species demonstrate bountiful growth and colonisation with a thick extracellular matrix surrounding the cell aggregates vastly different biofilm formation, when compared to their sparely grown single species biofilms which had been grown for the same length of time. It would be possible to conclude that the combination of the two species has a symbiotic effect on the attachment and growth of mixed species biofilms. In terms of enumeration, this is reflected in the increase in viable cell counts for *A. baumannii* which increased from 1.37 x 10^6 to 3.69 x 10^8 CFU/ml.

7.3.3 In vitro checkerboard assay

Erythromycin alone displayed little activity against both single species of *E. coli* ATCC 25922 and *A. baumannii* ATCC 19606 biofilm cultures, MIC values of 64 mg/L and 16 mg/L, respectively; whereas rifampicin when tested alone shows greater activity against both biofilm cultures, MIC values of 4 mg/L and 8 mg/L, respectively.

Synergy between both erythromycin and rifampicin in combination with colistin, was observed in the biofilm culture when determined in the checkerboard MIC assays. The combination of rifampicin and colistin was highly active against the *A. baumannii* 19606 biofilm infection with an FICI of 0.37 mg/L. The combination of erythromycin and colistin was also highly active against the biofilm cultures, also with an FICI of 0.37 mg/L. For *E. coli* ATCC 25922 biofilm cultures, the combination of rifampicin with colistin was found to be far more

effective treatment with an FICI value of 0.137. Erythromycin in combination with colistin was also found to be synergist with FICI value of 0.37. Checkerboard assays for the mixed species biofilms were not performed.

7.3.4 In vivo evaluation of treatment

7.3.4.1 Biofilm kill kinetics

Biofilms grown on sutures were quantified as 10^6 CFU/suture before injection and larval groups that have been infected in this way had a survival rate of (22.92% ± 3.61 Standard Deviation Percentage Points, [sdp]) after 96 hours. Figure 7.4 represents results from a single pathogenicity test.

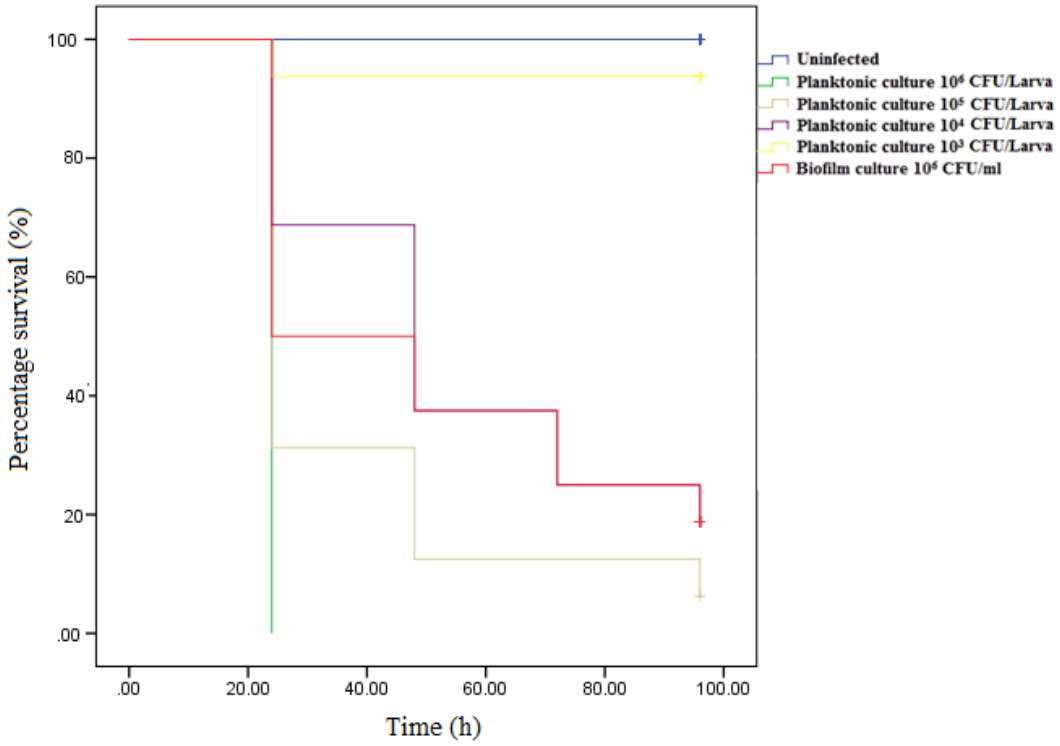


Figure 7.4: Kaplan Meier survival curve comparing the pathogenicity of planktonic cultures at varying CFU/ml concentrations (10^3 – 10^6 CFU/Larva) and biofilm culture (10^6 CFU/suture) of *Acinetobacter baumannii* ATCC 19606 over a 96 hour test period. Graph represents results from a single test.

As previously discussed, biofilm infections are generally considered to be more severe so why are these results showing the severity/pathogenicity of the biofilm infection to be less than that of its planktonic counterpart. After 24 hours, some of the larva showed signs of melanisation but their activity levels remained high. One explanation for this could be the induced immune response stimulated by the insertion of the pipette tip into the larva's proleg. The intricate wound healing process, triggered after wounding, could explain the

presence of a high degree of melanisation which in this case did not always result in the larvals death as a greater number of larval survived infected via the suture model compared to the planktonic infection. The results could be explained by the larva's immune response, triggered by the pipette tip, which had begun to not only effectively plug the wound trauma caused by the insertion of the suture but also the attached biofilm.

For *E. coli* ATCC 25922, planktonic cultures were found highly pathogenic towards the *G. mellonella* larvae at levels greater than 10^5 CFU/Larva.

Biofilm grown *E. coli* were found to be highly pathogenic towards *G. mellonella* larva, with 0% survival after 24 hours in all test groups. *E. coli* ATCC 25922 was found to be more pathogenic in both planktonic and biofilm grown deliberate infections compared to *A. baumannii* ATCC 19606.

Mixed species biofilms containing both *A. baumannii* ATCC 19606 and *E. coli* ATCC 25922 species was also found to be highly pathogenic towards *G. mellonella* larva, with 0% survival after 24 hours in all test groups. The increase in pathogenicity in comparison to *A. baumannii* ATCC 19606 infections alone can be explained by the presence of *E. coli* ATCC 25922 bacteria in the mixed species biofilm. Data not shown.

7.3.4.2 Efficacy of antimicrobial therapies in vivo against single and mixed-species biofilm infections

Both planktonic evaluation and *in vitro* analysis showed the combination of either erythromycin or rifampicin with colistin to be significantly more active than either drug alone against deliberate lethal *A. baumannii* ATCC 19606 infections. This was also supported *in vitro,* with rifampicin combinations resulted in the highest percentage survival and therefore most active combination against *A. baumannii* biofilm-associated infection groups.

Culture	% *G.mellonella* Survival after 96 hours					
	No treatment	Colistin	Erythromycin	Rifampicin	Colistin-Erythromycin	Colistin-Rifampicin
E. coli biofilm	0	91.67±3.61	-	-	-	-
A. baumannii biofilm	33.33±7.22	33.33±7.22	27.08±9.55	41.67±9.55	47.92±9.55	72.92±3.61
Mixed-species biofilms	0	77.08±13.01	-	-	-	-

Table 7.3: antimicrobial treatment efficacy against single and mixed species biofilm-associated infections evaluated in a *Galleria mellonella* infection model

Following treatment of *A. baumannii* biofilm-associated infections with colistin alone, the survival rate was 33.33% (± 7.22 sdp). Compared with treatment in combination with rifampicin (72.92% ± 3.61 sdp) or erythromycin (47.92% ± 9.55 sdp) colistin combination therapy was significantly more active in treating ATCC 19606 lethal infections (t test, P = < 0.01 and P = 0.05 respectively).

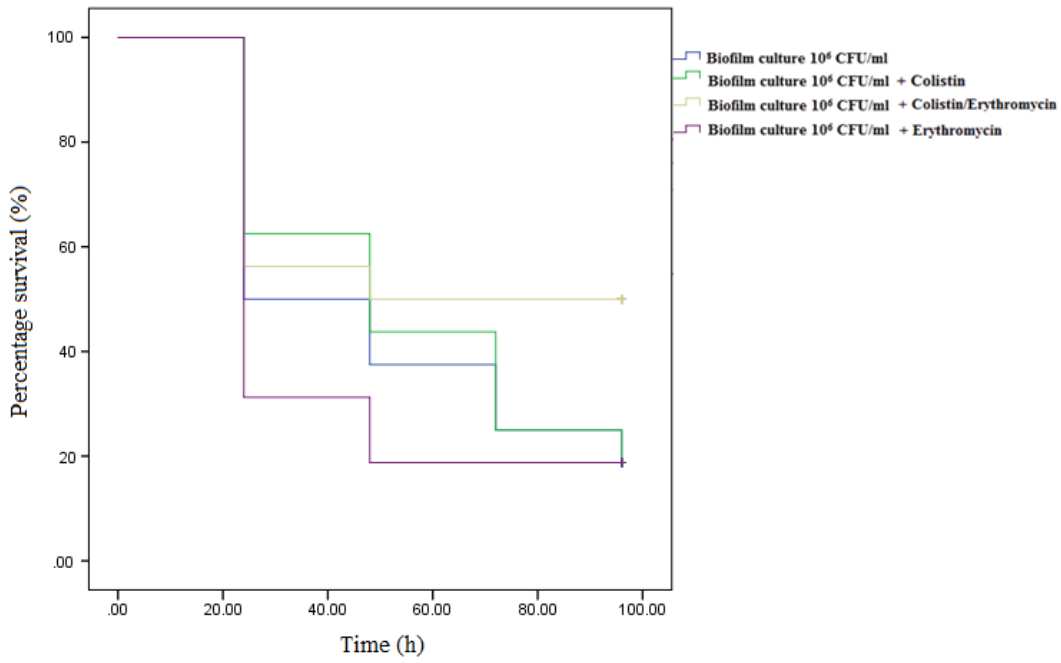


Figure 7.5: Kaplan Meier survival curves comparing the treatment of lethal biofilm *A. baumannii* ATCC 19606 infections with erythromycin and/or colistin alone and in combination. Survival was monitored every 24 hours over 96 hour period. Graph represents results from a single test.

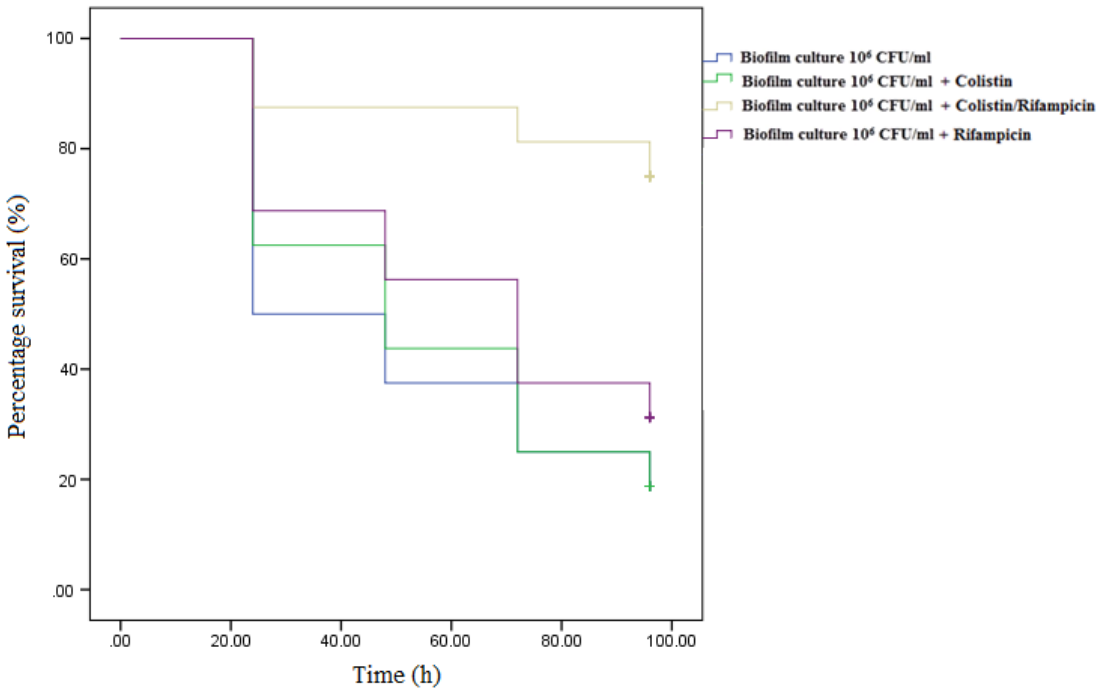


Figure 7.6: Kaplan Meier survival curves comparing the treatment of lethal biofilm *A. baumannii* ATCC 19606 infections with rifampicin and/or colistin alone and in combination. Survival was monitored every 24 hours over 96 hour period. Graph represents results from a single test.

Figure 7.5 and 7.6 shows a typical graphs of groups infected with biofilm grown cultures of ATCC 19606. It was unexpected that the pathogenicity of the biofilm-associated infection would be less lethal than that of the planktonic infection. However previously mentioned the larva's own immune response could have been working synergistically with the colistin combination therapy in a much more complex way which requires further investigation.

In regards to *E.coli* pathogenicity, the overall survival of the control groups deliberately infected with pathogenic levels of either planktonic or biofilm-associated infections was 0% at 24 hours for all test groups see table 7.1 and figure 7.7. Following treatment with colistin alone, the survival rate increased to 83.33±3.61% and 91.67±3.61% respectively. Due to the high survival rate of the larva with the treatment of colistin alone, it was deemed unnecessary to further treat with the either of the other agents or colistin combination therapy, as in this study, colistin combinations are the subject of interest therefore if the agent alone sufficiently treats the infection, the combination therapy becomes redundant. Also by minimising the number and amount of antimicrobial agents used, this reduces the risk of the overuse of antibiotics when it is not necessary, which could result in increased chances of acquired antibiotic resistance towards colistin and the other broad spectrum antibiotics used in this study.

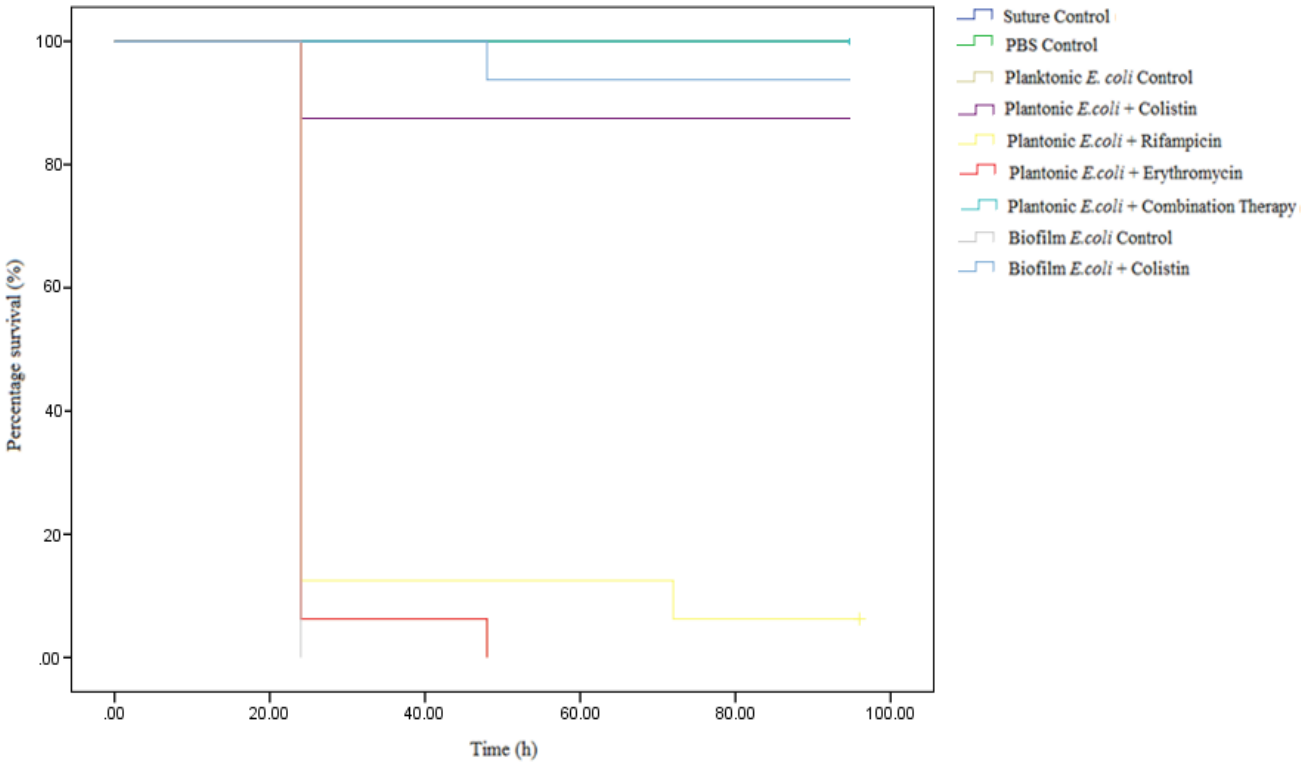


Figure 7.7: Kaplan Meier survival curve comparing the treatment of lethal planktonic and biofilm *E. coli* ATCC 25922 infections with either erythromycin, rifampicin and/or colistin alone and in combination. Survival was monitored every 24 hours over 96 hour period. Graph represents results from a single test.

Figure 7.7 shows a typical Kaplan Meier survival curve comparing the treatment of lethal planktonic and biofilm *E. coli* ATCC 25922 infections with either erythromycin, rifampicin and/or colistin alone and in combination. The p-value was found to be significant ($P<0.05$), therefore it is possible to conclude that the chances of survival is dependent on whether the test group received treatment.

It is quite possible that the growth rate of the biofilm-associated infection greatly influences the survival rate of the larva. Where treatment has been applied immediately after the suture was injected in to the larva, it is possible that not enough time has passed for the infection to establish itself within the model. Further investigation in to the influence of time dependant administration would shed light on how the infection establishes itself in the *G. mellonella* model.

Many studies have found a link between biofilm growth rates and susceptibility towards different antibiotics. DuGuid *et al.*[94] found that *Staphylococcus epidermidis* biofilm growth rates strongly influenced susceptibility; from their study, they concluded the faster the rate of cell growth, the more rapid the rate of inactivation by ciprofloxacin. A review by Donlan and Costerton[88] found other results supporting the idea that biofilm growth rates influence the susceptibility of the biofilm to antimicrobial agents with several different combinations of bacteria and antimicrobial agents. Therefore in the present study the rate at which the biofilm establishes itself within the *G. mellonella* model maybe influence the infections susceptibility to the agents tested.

7.3.4.2.1 Mixed species

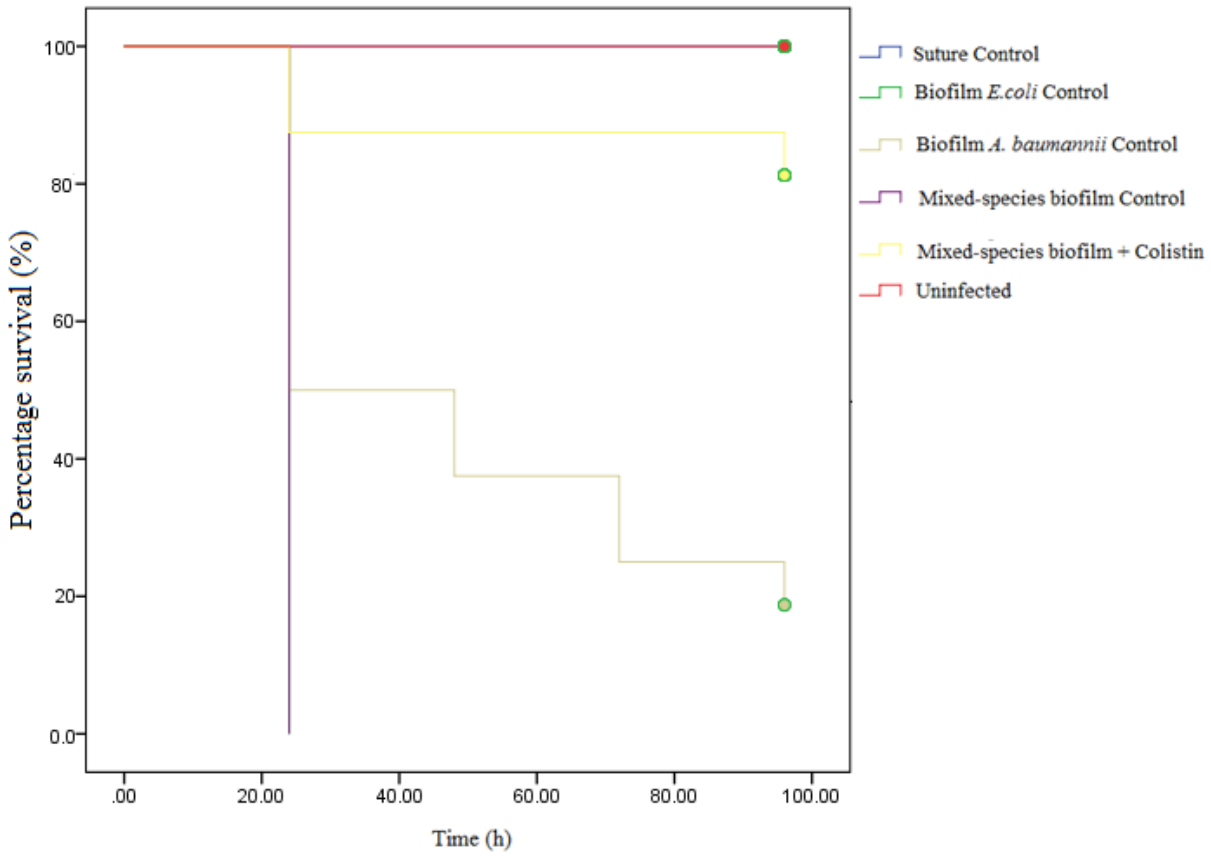


Figure 7.6: Kaplan Meier survival curve comparing single and mixed-species biofilms. Survival was monitored every 24 hours over 96 hour period. Graph represents results from a single test.

Interestingly monotherapy treatment of colistin against the lethal mixed species biofilm associated infections resulted in average survival of 77.08±13.01, see table 7.3, which was found to be significantly higher than if the groups were to receive no treatment. Due to the high survival rate of the larva with the treatment of colistin alone, it was deemed unnecessary to further treat with the colistin combination therapy, therefore the other agents and combination therapies were not tested. This is a very interesting result as previous *in vitro*

analysis had not indicated towards this outcome. One possible explanation for this result could be that *E. coli* bacteria are controlling the pathogenicity of the mixed species biofilm through biofilm mechanism through quorum sensing (cell to cell communication). Quorum sensing as defined by Miller and Bassler[95], is 'the regulation of gene expression in response to fluctuations in cell-population density. Bacteria use quorum sensing communication to regulate a diverse array of physiological activities including, but not exclusive, bioluminescence, virulence and biofilm formation and dispersal. This cell to cell communication system occurs both within and between bacterial species, thereby coordinating group behaviour, critical for the survival and development of biofilms, including extracellular polymer production[37, 95-97].

In the present study, the cell signalling could be controlling the virulence of the mixed species biofilm, so that the virulence is infection is caused only by *E. coli* bacterial cells, which has previously been shown to be effectively treated with colistin alone.

The explanation above does however raise future concerns about long term treatment of mixed species biofilm-associated infections due to the possible outcome of the 'dormant' *A. baumannii* cells becoming what is known as persister cells[83]. What is interesting about persister cells is that they are not just simply resistant to the antibiotics employed to treat the infection, rather there dormancy state allows them to recover the infection once the threshold of cells required to trigger quorum sensing has no longer been reached. In order words the dormant cells are able to recover the pathogenicity of the biofilm after treatment has removed the infective actively growing cells[83]. This raises long term concerns and therefore it would be interesting to see what would happen to the *G. mellonella* test groups

over a longer test period and see whether the infection would establish itself within the model later on in the study.

7.4 SUMMARY OF RESULTS

Although planktonically grown *A baumannii* produces an acute lethal effect at high numbers (10^5/10^6 CFU, Figure 4) the biofilm induced model in *G. mellonella* is probably a more realistic representation of infections that occur in nosocomial environments than direct inoculation with planktonic growth phase organisms. Also previously mentioned the larva's own immune response could have been working synergistically with the colistin combination therapy in a much more complex way which requires further investigation.

Treatment of colistin alone against the lethal mixed species biofilm associated infections resulted in 100% survival which could be as a result of *E. coli* bacteria are controlling the pathogenicity of the mixed species biofilm through mechanisms of quorum sensing (cell to cell communication). Comparing the single species results, *E. coli* biofilms were effectively treated with colistin alone, whereas there was a higher rate of survival in the groups treated with either colistin combination compared to treatment with colistin alone. It could be possible that *E. coli* cells are able to trigger the *A. baumannii* cells to become dormant resulting in the infection only displaying and exhibiting *E. coli* pathogenic traits, which was previously shown to be effectively treated with colistin alone however further analysis would be required to establish whether this is true.

8 GENERAL DISCUSSION

The optimal treatment for *A. baumannii*, especially for those infections cause by multidrug resistant strains, still remains to be established. This study explored the use of combining old antibiotics, to which the bacteria are still susceptible to, with broad spectrum antibiotics to investigate if the agents together have a synergist effect on the treatment infections cause by MDR *A. baumannii*.

Previous studies report that colistin when tested *in vitro* initially displays bactericidal activity against clinical strains of *A.baumannii* but this appears to be overcome after 24 hours[15]. The present study agrees that colistin does not possess sufficient bactericidal activity to overcome initial *A. baumannii* infections in the first phase of treatment. Since colistin demonstrates excellent synergy *in vitro* with the two tested BSA, and larval survival is significantly increased in the combination model of infection, suggesting there is sufficient bactericidal activity within the combinations that is able to overcome the infection.

Cai *et al.*[16] analysed antimicrobial strategies to avoid colistin resistance of *A. baumannii* comparing a number of patient studies evaluating combination therapies of colistin with a range of antibiotics, including rifampicin. Rifampicin/colistin combinations were shown *in vitro, in vivo* and in clinical reports to be promising therapeutic options against MDR *A. baumannii* infections. The model evaluated in the present study supports these finding and also provides evidence on the use of the rifampicin/colistin combination therapy against *A. baumannii* biofilm infections in a simple, cheap and ethically acceptable model.

Other macrolide antibiotics in combination with colistin have been shown previously to be synergists *in vitro*; Timurkaynak *et al.*[79] found azithromycin in combination with colistin to be synergistic against three different *A. baumannii* isolates. In the present study, the macrolide erythromycin was found to both be synergist *in vitro* and significantly improved survival of the *G. mellonella.*

Although planktonic grown *A baumannii* produces an acute lethal effect at high numbers (10^5/10^6 CFU, Figure 4) the biofilm induced model in *G. mellonella* is probably a more realistic representation of infections that occur in nosocomial environments than direct inoculation with planktonic grown organisms.

Interestingly treatment of synergistic combinations of colistin with either erythromycin or rifampicin against biofilm associated infections in *G. mellonella* is considerably more effective than with planktonic grown cells increased survival of those infected with the biofilm culture of 19606 show that the biofilm has induced the host immune system to a point where it is beginning to successful fight the infection. The biofilm grown organisms produced a less aggressive course of infection which may be due to modulation by the host defence peptides against a more localised infection within *G. mellonella.*

8.1 CONCLUSIONS

The work presented here provides positive *in vivo* model evidence of the efficacy of colistin as an effective synergist to treat serious infections in humans by *A. baumannii*. The combination of specific broad spectrum antibiotics shown to be synergistic with low concentrations of colistin may be useful drugs in patients. Effective combination therapy with

colistin could reduce the emergence of resistance and heteroresistance of *A. baumannii* to colistin.

Treatment of colistin alone against the lethal mixed species biofilm associated infections resulted in 100% survival. This is a very interesting result as previous *in vitro* analysis had not indicated towards this outcome. One possible explanation for this result could be that *E. coli* bacteria are controlling the pathogenicity of the mixed species biofilm through biofilm mechanism through quorum sensing (cell to cell communication) which results in the *A. baumannii* cells becoming dormant therefore the infection only displays *E. coli* pathogenic traits, which has previously been shown to be effectively treated with colistin alone.

9 FURTHER WORK

This work provides positive *in vivo* model evidence of the efficacy of colistin as an effective synergist to treat serious infections in humans by *A. baumannii*. The combination of specific broad spectrum antibiotics shown to be synergistic with low concentrations of colistin may be useful drugs in patients. Effective combination therapy with colistin could reduce the emergence of resistance and heteroresistance of *A. baumannii* to colistin. Therefore further work should be carried out to investigate minimum effective levels of the combination therapy against such prevalent nosocomial infections. Further work should also investigate the relationship between *E. coli* and *A. baumannii* species in regards to their pathogenicity traits within a mixed species biofilm.

Highlighted within the literature was the lack of knowledge and understanding of the antimicrobial agents belonging to the Polymyxin family. Do to the drug being 'abandoned' during the 1950s, the agents have not undergone the same rigorous development procedures that other antimicrobial agents have. Therefore further work should be carried out investigating the pharmacokinetics and pharmacodynamics of this last resort antimicrobial family in order to maximise the activity of the drugs and also minimise the risk of antimicrobial resistance towards the drugs.

Within the work carried out here, it was apparent that certain techniques had associated inherent errors within the method. In particular the stripping of the biofilm from the surgical suture in order to enumerate the number of total viable cells attached to the surface. Many assumptions are made when using this technique, such no cells were damaged during the vortexing procedure and therefore the number counted on the agar plates is the 'true'

number of viable cells. Also it is assumed that all cells are striped from the suture surface. One method which could be utilised to quantify the number of bacterial cells attached to the suture surface which possess high accuracy and precision is qPCR. By fluorescently tagging bacterial cells, and monitoring the increased signal and comparing to a calibration graph of known cell numbers it would be possible to accurately quantify the number of bacterial cells attached to the suture surface and this would prevent the inherent errors caused by the methods of quantification employed in this study.

From the work carried out in this study, it suggested that that the growth rate of the biofilm-associated infection greatly influences the survival rate of the larva. Where treatment has been applied immediately after the suture was injected in to the larva, it is possible that not enough time has passed for the infection to establish itself within the model. Further investigation would help establish the relationship between biofilm formation and growth within the *Galleria mellonella* model and is susceptibility towards certain antimicrobial agents.

10 REFERENCES

1. Giamarellou, H., Antoniadou, A. & Kanellakopoulou, K. Acinetobacter baumannii: a universal threat to public health? *International Journal of Antimicrobial Agents* **32**, 106-119 (2008).
2. Enoch, D.A., Birkett, C.I. & Ludlam, H.A. Non-fermentative Gram-negative bacteria. *International Journal of Antimicrobial Agents* **29, Supplement 3**, S33-S41 (2007).
3. Jones, A., Morgan, D., Walsh, A., Turton, J., Livermore, D., Pitt, T., Green, A., Gill, M. and Mortiboy D. Importation of multidrug-resistant Acinetobacter baumannii spp infections with casualties from Iraq. *Lancet Infectious Diseases* **6**, 2630 - 2634 (2006).
4. Neonakis, I.K., Spandidos, D.A. & Petinaki, E. Confronting multidrug-resistant Acinetobacter baumannii: a review. *International Journal of Antimicrobial Agents* **37**, 102-109 (2011).
5. Peleg, A.Y., Seifert, H. & Paterson, D.L. Acinetobacter baumannii: Emergence of a Successful Pathogen. *Clinical Microbiology Reviews* **21**, 538-582 (2008).
6. Vaneechoutte, M., Dijkshoorn, L., Nemec, A., K A., , P., Wauters, G. in Manual of Clinical Microbiology, Edn. 11th (ed. P.R. Murray, Baron, E. J., Pfaller, M. A., Tenover, F. C. and Ypolken, R. H.) (2011).
7. Zanetti, G., Blanc, D., Federli, I., Raffoul, W., Petignat, C. Importation of Acinetobacter baumannii Into a Burn Unit: A Recurrent Outbreak of Infection Associated with widespread environmental contamination. *Infectious Control Hospital Epidemiology* **28**, 723-725 (2007).
8. Falagas M.E., R., P.I, Matthaiou, D.K., Virtzili S., Nikita D. and Michalopoulos, A. Pandrug-resistant Klebsiella pneumoniae, Pseudomonas aerginosa and Acinetobacter baumannii infections: Characteristics and outcome in a series of 28 patients. *Clinical Infectious Diseases* **40**, 1333- 1341 (2008).
9. Kardas P. Devine, S.G., A. and Roberts, C. A systematic review and meta-analysis of the misuse of antibiotic therapies in the community *International Journal of Antimicrobial Agents* **26**, 106 -113 (2005).
10. McDonnell Norms, G. Antibiotic Overuse: The Influence of Social Norms. *Journal of the American College of Surgeons* **207**, 265-275 (2008).
11. Fishbain, J. & Peleg, A.Y. Treatment of Acinetobacter Infections. *Clinical Infectious Diseases* **51**, 79-84 (2010).
12. Hawley, J.S., Murray, C.K. & Jorgensen, J.H. Colistin Heteroresistance in Acinetobacter and Its Association with Previous Colistin Therapy. *Antimicrobial Agents and Chemotherapy* **52**, 351-352 (2008).
13. Hornsey, M. & Wareham, D.W. In Vivo Efficacy of Glycopeptide-Colistin Combination Therapies in a Galleria mellonella Model of Acinetobacter baumannii Infection. *Antimicrobial Agents and Chemotherapy* **55**, 3534-3537 (2011).
14. Mihu, M.R. & Martinez, L.R. Novel therapies for treatment of multi-drug resistant Acinetobacter baumannii skin infections. *Virulence* **2**, 97-102 (2011).
15. Hornsey, M., Phee, L., Longshaw, C. & Wareham, D.W. In vivo efficacy of telavancin/colistin combination therapy in a Galleria mellonella model of Acinetobacter baumannii infection. *International Journal of Antimicrobial Agents* **41**, 285-287 (2013).

16. Cai, Y., Chai, D., Wang, R., Liang, B. & Bai, N. Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. *Journal of Antimicrobial Chemotherapy (JAC)* **67**, 1607-1615 (2012).
17. Kavanagh, K. & Reeves, E.P. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. *FEMS microbiology reviews* **28**, 101-112 (2004).
18. Aperis, G. et al. Galleria mellonella as a model host to study infection by the Francisella tularensis live vaccine strain. *Microbes and Infection* **9**, 729-734 (2007).
19. Apidianakis, Y. et al. Challenge of Drosophila melanogaster with Cryptococcus neoformans and Role of the Innate Immune Response. *Eukaryotic Cell* **3**, 413-419 (2004).
20. Cotter, G., Doyle, S. & Kavanagh, K. Development of an insect model for the in vivo pathogenicity testing of yeasts. *FEMS immunology and medical microbiology* **27**, 163-169 (2000).
21. Jander, G., Rahme, L.G. & Ausubel, F.M. Positive Correlation between Virulence of Pseudomonas aeruginosa Mutants in Mice and Insects. *Journal of Bacteriology* **182**, 3843-3845 (2000).
22. Mylonakis, E. et al. Galleria mellonella as a Model System To Study Cryptococcus neoformans Pathogenesis. *Infection and Immunity* **73**, 3842-3850 (2005).
23. Peleg, A.Y. et al. Galleria mellonella as a model system to study Acinetobacter baumannii pathogenesis and therapeutics. *Antimicrob Agents Chemother* **53**, 2605-2609 (2009).
24. Salzet, M. Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. *Trends in Immunology* **22**, 285-288 (2001).
25. Bergogne-Bérézin, E. & Towner, K.J. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clinical Microbiology Reviews* **9**, 148-165 (1996).
26. Howard, A., O'Donoghue, M., Feeney, A. & Sleator, R.D. Acinetobacter baumannii. *Virulence* **3**, 243-250 (2012).
27. Coelho, J.M. et al. Occurrence of Carbapenem-Resistant Acinetobacter baumannii Clones at Multiple Hospitals in London and Southeast England. *Journal of Clinical Microbiology* **44**, 3623-3627 (2006).
28. Abbo, A. et al. Multidrug-resistant Acinetobacter baumannii. *Emerg Infect Dis* **11**, 22-29 (2005).
29. Poole, K. Multidrug resistance in Gram-negative bacteria. *Current Opinion in Microbiology* **4**, 500-508 (2001).
30. Vaara, M. Agents that increase the permeability of the outer membrane. *Microbiological Reviews* **56**, 395-411 (1992).
31. Biswas, S., Brunel, J.M., Dubus, J.C., Reynaud-Gaubert, M. & Rolain, J.M. Colistin: an update on the antibiotic of the 21st century. *Expert review of anti-infective therapy* **10**, 917-934 (2012).
32. Rolain, J.-M., Roch, A., Castanier, M., Papazian, L. & Raoult, D. Acinetobacter baumannii Resistant to Colistin With Impaired Virulence: A Case Report From France. *Journal of Infectious Diseases* **204**, 1146-1147 (2011).
33. Donlan, R.M. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases* **33**, 1387-1392 (2001).

34. Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. & Lappin-Scott, H.M. Microbial biofilms. *Annual review of microbiology* **49**, 711-745 (1995).
35. Branda, S.S., Vik, S., Friedman, L. & Kolter, R. Biofilms: the matrix revisited. *Trends in microbiology* **13**, 20-26 (2005).
36. Davey, M.E. & O'Toole, G.A. Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiology and Molecular Biology Reviews* **64**, 847-867 (2000).
37. Donlan, R.M. Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**, 881-890 (2002).
38. Flemming, H.-C. & Wingender, J. The biofilm matrix. *Nature Reviews Microbiology* **8**, 623-633 (2010).
39. Hunter, P. The mob response. The importance of biofilm research for combating chronic diseases and tackling contamination. *EMBO reports* **9**, 314-317 (2008).
40. Costerton, J.W.S.P.S.G.E.P. Bacterial Biofilms: A Common Cause of Persistent Infections. (cover story). *Science* **284**, 1318-1322 (1999).
41. Stewart, P.S. & Costerton, J.W. Antibiotic resistance of bacteria in biofilms. *Lancet (London, England)* **358**, 135-138 (2001).
42. Percival, S.L. et al. A review of the scientific evidence for biofilms in wounds. *Wound Repair and Regeneration* **20**, 647-657 (2012).
43. Rodríguez-Baño, J. et al. Biofilm formation in Acinetobacter baumannii: associated features and clinical implications. *Clinical Microbiology and Infection* **14**, 276-278 (2008).
44. Kassamali, Z., Rotschafer, J.C., Jones, R.N., Prince, R.A. & Danziger, L.H. Polymyxins: wisdom does not always come with age. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **57**, 877-883 (2013).
45. Gales, A.C., Jones, R.N. & Sader, H.S. Contemporary activity of colistin and polymyxin B against a worldwide collection of Gram-negative pathogens: results from the SENTRY Antimicrobial Surveillance Program (2006-09). *The Journal of antimicrobial chemotherapy* **66**, 2070-2074 (2011).
46. Betts, J.W., Phee, L.M., Woodford, N. & Wareham, D.W. Activity of colistin in combination with tigecycline or rifampicin against multidrug-resistant Stenotrophomonas maltophilia. *Eur J Clin Microbiol Infect Dis* **33**, 1565-1572 (2014).
47. Li, J., Nation, R.L., Milne, R.W., Turnidge, J.D. & Coulthard, K. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *International Journal of Antimicrobial Agents* **25**, 11-25 (2005).
48. Koike, M., Iida, K. & Matsuo, T. Electron microscopic studies on mode of action of polymyxin. *Journal of Bacteriology* **97**, 448-452 (1969).
49. Sud, I.J. & Feingold, D.S. Detection of Agents That Alter the Bacterial Cell Surface. *Antimicrobial Agents and Chemotherapy* **8**, 34-37 (1975).
50. Bauman, R.W. Microbiology: With Diseases by Taxonomy. (Pearson/Benjamin Cummings, 2013).
51. Loh, J.M., Adenwalla, N., Wiles, S. & Proft, T. Galleria mellonella larvae as an infection model for group A streptococcus. *Virulence* **4**, 419-428 (2013).
52. Mukherjee, K. et al. Galleria mellonella as a Model System for Studying Listeria Pathogenesis. *Applied and Environmental Microbiology* **76**, 310-317 (2010).
53. Schrama, D., Helliwell, N., Neto, L. & Faleiro, M.L. Adaptation of Listeria monocytogenes in a simulated cheese medium: effects on virulence using the Galleria mellonella infection model. *Letters in applied microbiology* **56**, 421-427 (2013).

54. Harding, C.R. et al. Legionella pneumophila pathogenesis in the Galleria mellonella infection model. *Infect Immun* **80**, 2780-2790 (2012).
55. Arvanitis, M., Glavis-Bloom, J. & Mylonakis, E. Invertebrate models of fungal infection. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1832**, 1378-1383 (2013).
56. Bergin, D., Murphy, L., Keenan, J., Clynes, M. & Kavanagh, K. Pre-exposure to yeast protects larvae of Galleria mellonella from a subsequent lethal infection by Candida albicans and is mediated by the increased expression of antimicrobial peptides. *Microbes and infection / Institut Pasteur* **8**, 2105-2112 (2006).
57. Fuchs, B.B. et al. Role of filamentation in Galleria mellonella killing by Candida albicans. *Microbes and Infection* **12**, 488-496 (2010).
58. Fedhila, S. et al. Comparative analysis of the virulence of invertebrate and mammalian pathogenic bacteria in the oral insect infection model Galleria mellonella. *Journal of invertebrate pathology* **103**, 24-29 (2010).
59. Smith, T.L. External Morphology of the Larva, Pupa, and Adult of the Wax Moth, Galleria mellonella L. *Journal of the Kansas Entomological Society* **38**, 287-310 (1965).
60. Browne, N., Heelan, M. & Kavanagh, K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. *Virulence* **4**, 597-603 (2013).
61. Krautz, R., Arefin, B. & Theopold, U. Damage signals in the insect immune response. *Frontiers in Plant Science* **5**, 342 (2014).
62. Boguś, M.I. et al. Different defense strategies of Dendrolimus pini, Galleria mellonella, and Calliphora vicina against fungal infection. *Journal of Insect Physiology* **53**, 909-922 (2007).
63. Rowley, A.F. & Ratcliffe, N.A. A histological study of wound healing and hemocyte function in the wax-moth Galleria mellonella. *Journal of Morphology* **157**, 181-199 (1978).
64. testing, E.C.o.A.S. in Breakpoint tables, Vol. 2012 (EUCAST 2012).
65. Perez, F. et al. Global Challenge of Multidrug-Resistant Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy* **51**, 3471-3484 (2007).
66. Behera, B. et al. Evaluation of susceptibility testing methods for polymyxin. *International Journal of Infectious Diseases* **14**, e596-e601 (2010).
67. Galani, I. et al. Colistin susceptibility testing by Etest and disk diffusion methods. *International Journal of Antimicrobial Agents* **31**, 434-439 (2008).
68. Ramarao, N., Nielsen-Leroux, C. & Lereclus, D. The Insect Galleria mellonella as a Powerful Infection Model to Investigate Bacterial Pathogenesis. *Journal of Visualized Experiments : JoVE*, 4392 (2012).
69. Wand, M.E., Bock, L.J., Turton, J.F., Nugent, P.G. & Sutton, J.M. Acinetobacter baumannii virulence is enhanced in Galleria mellonella following biofilm adaptation. *Journal of Medical Microbiology* **61**, 470-477 (2012).
70. Jorgensen, J.H. & Ferraro, M.J. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases* **49**, 1749-1755 (2009).
71. (ESCMID), E.C.f.A.S.T.E.o.t.E.S.o.C.M.a.I.D. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection* **9**, 1-7 (2003).

72. Odds, F.C. Synergy, antagonism, and what the chequerboard puts between them. *The Journal of antimicrobial chemotherapy* **52**, 1 (2003).
73. ESCMID, Vol. 2014 (ESCMID, www.escmid.org/research_projects/escmid_conferences/past_escmid_conferences/reviving_old_antibiotics; 2014).
74. Hsieh, M.H., Yu, C.M., Yu, V.L. & Chow, J.W. Synergy assessed by checkerboard a critical analysis. *Diagnostic Microbiology and Infectious Disease* **16**, 343-349 (1993).
75. Pimentel, M. et al. Low-dose nocturnal tegaserod or erythromycin delays symptom recurrence after treatment of irritable bowel syndrome based on presumed bacterial overgrowth. *Gastroenterology & hepatology* **5**, 435-442 (2009).
76. Diacon, A. et al. Early bactericidal activity of high-dose rifampin in patients with pulmonary tuberculosis evidenced by positive sputum smears. *Antimicrobial agents and chemotherapy* **51**, 2994-2996 (2007).
77. Barie, P.S. Antibiotic-resistant gram-positive cocci: implications for surgical practice. *World journal of surgery* **22**, 118-126 (1998).
78. Healy, J.K., P.J. Drumt, and A.J. Elliott Kanamycin Dosage in Renal Failure. *Australian and New Zealand Journal of Medicine* **3**, 474-479 (1973).
79. Timurkaynak, F. et al. In vitro activities of non-traditional antimicrobials alone or in combination against multidrug-resistant strains of Pseudomonas aeruginosa and Acinetobacter baumannii isolated from intensive care units. *Int J Antimicrob Agents* **27**, 224-228 (2006).
80. Reysenbach, A.-L. & Cady, S.L. Microbiology of ancient and modern hydrothermal systems. *Trends in microbiology* **9**, 79-86 (2001).
81. Stoodley, P., Sauer, K., Davies, D. G., Costerton, J. W. Biofilms as Complex Differentiated Communities. *Annual review of microbiology* **56**, 187-209 (2002).
82. Hall-Stoodley, L., Costerton, J.W. & Stoodley, P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nat Rev Micro* **2**, 95-108 (2004).
83. Lewis, K. Persister cells, dormancy and infectious disease. *Nat Rev Micro* **5**, 48-56 (2007).
84. Bryers, J.D. Medical biofilms. *Biotechnology and Bioengineering* **100**, 1-18 (2008).
85. Gubner, R. & Beech, I.B. The effect of extracellular polymeric substances on the attachment of Pseudomonas NCIMB 2021 to AISI 304 and 316 stainless steel. *Biofouling* **15**, 25-36 (2000).
86. Scheuerman, T.R., Camper, A.K. & Hamilton, M.A. Effects of Substratum Topography on Bacterial Adhesion. *Journal of colloid and interface science* **208**, 23-33 (1998).
87. Robitaille, G., Choiniere, S., Ells, T., Deschenes, L. & Mafu, A.A. Attachment of Listeria innocua to polystyrene: effects of ionic strength and conditioning films from culture media and milk proteins. *Journal of food protection* **77**, 427-434 (2014).
88. Donlan, R.M. & Costerton, J.W. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews* **15**, 167-167 (2002).
89. Kim, J.K. & Harrison, M.A. Surrogate selection for Escherichia coli O157: H7 based on cryotolerance and attachment to romaine lettuce. *Journal of Food Protection®* **72**, 1385-1391 (2009).
90. Quintavalla, S. & Vicini, L. Antimicrobial food packaging in meat industry. *Meat Science* **62**, 373-380 (2002).

91. Christensen, G.D. et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology* **22**, 996-1006 (1985).
92. Harrison, J.J. et al. The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the Calgary Biofilm Device. *Biological Procedures Online* **8**, 194-215 (2006).
93. Rao, S. in microrao, Vol. 2012. (ed. S. Rao) (Sridhar Rac P.N., http://www.microrao.com/micronotes/pg/Gram%20stain.pdf; 2012).
94. Duguid, I., Evans, E., Brown, M. & Gilbert, P. Growth-rate-independent killing by ciprofloxacin of biofilm-derived Staphylococcus epidermidis evidence for cell-cycle dependency. *Journal of antimicrobial chemotherapy* **30**, 791-802 (1992).
95. Miller, M.B. & Bassler, B.L. Quorum sensing in bacteria. *Annual review of microbiology* **55**, 165-199 (2001).
96. Nadell, C.D., Xavier, J.B., Levin, S.A. & Foster, K.R. The evolution of quorum sensing in bacterial biofilms. *PLOS BIOLOGY* **6**, 171-179 (2008).
97. Higgins, D.A. et al. The major Vibrio cholerae autoinducer and its role in virulence factor production. *Nature* **450**, 883-886 (2007).

11 APPENDIX

11.1 SECTION 4

11.1.1 ATCC 19606 Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Uninfected Control	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
Planktonic 10^6 CFU/Larva	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
Planktonic 10^5 CFU/Larva	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.313	.116	11	5
	12	48.000	Complete	.	.	12	4
	13	48.000	Complete	.	.	13	3
	14	48.000	Complete	.125	.083	14	2
	15	96.000	Complete	.063	.061	15	1
	16	96.000	Censored	.	.	15	0
Planktonic 10^4 CFU/Larva	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.688	.116	5	11
	6	48.000	Complete	.	.	6	10
	7	48.000	Complete	.	.	7	9
	8	48.000	Complete	.	.	8	8
	9	48.000	Complete	.438	.124	9	7
	10	72.000	Complete	.	.	10	6
	11	72.000	Complete	.313	.116	11	5
	12	96.000	Complete	.250	.108	12	4
	13	96.000	Censored	.	.	12	3
	14	96.000	Censored	.	.	12	2
	15	96.000	Censored	.	.	12	1
	16	96.000	Censored	.	.	12	0
Planktonic 10^3 CFU/Larva	1	24.000	Complete	.938	.061	1	15
	2	96.000	Censored	.	.	1	14
	3	96.000	Censored	.	.	1	13
	4	96.000	Censored	.	.	1	12
	5	96.000	Censored	.	.	1	11
	6	96.000	Censored	.	.	1	10
	7	96.000	Censored	.	.	1	9
	8	96.000	Censored	.	.	1	8
	9	96.000	Censored	.	.	1	7
	10	96.000	Censored	.	.	1	6
	11	96.000	Censored	.	.	1	5
	12	96.000	Censored	.	.	1	4
	13	96.000	Censored	.	.	1	3
	14	96.000	Censored	.	.	1	2
	15	96.000	Censored	.	.	1	1
	16	96.000	Censored	.	.	1	0

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	71.665	4	.000

Test of equality of survival distributions for the different levels of Group.

11.1.2 AYE Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Uninfected	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
Original O/N culture	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-1 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-2 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.188	.098	13	3
	14	48.000	Complete	.125	.083	14	2
	15	96.000	Complete	.063	.061	15	1
	16	96.000	Censored	.	.	15	0
10-3 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.625	.121	6	10
	7	48.000	Complete	.	.	7	9
	8	48.000	Complete	.500	.125	8	8
	9	96.000	Censored	.	.	8	7
	10	96.000	Censored	.	.	8	6
	11	96.000	Censored	.	.	8	5
	12	96.000	Censored	.	.	8	4
	13	96.000	Censored	.	.	8	3
	14	96.000	Censored	.	.	8	2
	15	96.000	Censored	.	.	8	1
	16	96.000	Censored	.	.	8	0

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	61.261	4	.000

Test of equality of survival distributions for the different levels of Group.

11.1.3 W Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Uninfected	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
Original O/N culture	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-1 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-2 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.063	.061	15	1
	16	96.000	Censored	.	.	15	0
10-3 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.063	.061	15	1
	16	96.000	Censored	.	.	15	0

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	68.382	4	.000

Test of equality of survival distributions for the different levels of Group.

11.1.4 A14 Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Original O/N culture	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-1 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.125	.083	14	2
	15	48.000	Complete	.063	.061	15	1
	16	72.000	Complete	.000	.000	16	0
10-2 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.500	.125	8	8
	9	48.000	Complete	.	.	9	7
	10	48.000	Complete	.	.	10	6
	11	48.000	Complete	.313	.116	11	5
	12	72.000	Complete	.	.	12	4
	13	72.000	Complete	.188	.098	13	3
	14	96.000	Complete	.125	.083	14	2
	15	96.000	Censored	.	.	14	1
	16	96.000	Censored	.	.	14	0
10-3 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.625	.121	6	10
	7	48.000	Complete	.	.	7	9
	8	48.000	Complete	.	.	8	8
	9	48.000	Complete	.	.	9	7
	10	48.000	Complete	.375	.121	10	6
	11	72.000	Complete	.313	.116	11	5
	12	96.000	Complete	.250	.108	12	4
	13	96.000	Censored	.	.	12	3
	14	96.000	Censored	.	.	12	2
	15	96.000	Censored	.	.	12	1
	16	96.000	Censored	.	.	12	0

Means and Medians for Survival Time

Group	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Original O/N culture	24.000	.000	24.000	24.000	24.000	.	.	.
10-1 dilution	28.500	3.263	22.104	34.896	24.000	.	.	.
10-2 dilution	48.000	7.301	33.690	62.310	24.000	.	.	.
10-3 dilution	55.500	7.902	40.013	70.987	48.000	11.619	25.227	70.773
Overall	39.000	3.187	32.753	45.247	24.000	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	20.514	3	.000

Test of equality of survival distributions for the different levels of Group.

11.1.5 A17 Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Original O/N culture	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-1 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.500	.125	8	8
	9	48.000	Complete	.	.	9	7
	10	48.000	Complete	.	.	10	6
	11	48.000	Complete	.313	.116	11	5
	12	72.000	Complete	.250	.108	12	4
	13	96.000	Complete	.188	.098	13	3
	14	96.000	Censored	.	.	13	2
	15	96.000	Censored	.	.	13	1
	16	96.000	Censored	.	.	13	0
10-2 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.500	.125	8	8
	9	48.000	Complete	.	.	9	7
	10	48.000	Complete	.375	.121	10	6
	11	96.000	Complete	.313	.116	11	5
	12	96.000	Censored	.	.	11	4
	13	96.000	Censored	.	.	11	3
	14	96.000	Censored	.	.	11	2
	15	96.000	Censored	.	.	11	1
	16	96.000	Censored	.	.	11	0
10-3 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.813	.098	3	13
	4	48.000	Complete	.	.	4	12
	5	48.000	Complete	.688	.116	5	11
	6	96.000	Complete	.625	.121	6	10
	7	96.000	Censored	.	.	6	9
	8	96.000	Censored	.	.	6	8
	9	96.000	Censored	.	.	6	7
	10	96.000	Censored	.	.	6	6
	11	96.000	Censored	.	.	6	5
	12	96.000	Censored	.	.	6	4
	13	96.000	Censored	.	.	6	3
	14	96.000	Censored	.	.	6	2
	15	96.000	Censored	.	.	6	1
	16	96.000	Censored	.	.	6	0

Means and Medians for Survival Time

Group	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Original O/N culture	24.000	.000	24.000	24.000	24.000	.	.	.
10-1 dilution	49.500	7.796	34.219	64.781	24.000	.	.	.
10-2 dilution	54.000	8.759	36.832	71.168	24.000	.	.	.
10-3 dilution	76.500	8.123	60.579	92.421	.	.	.	.
Overall	51.000	4.136	42.893	59.107	24.000	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	24.072	3	.000

Test of equality of survival distributions for the different levels of Group.

11.1.6 A13 Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Original O/N culture	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
10-1 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.188	.098	13	3
	14	48.000	Complete	.125	.083	14	2
	15	72.000	Complete	.063	.061	15	1
	16	98.000	Complete	.000	.000	16	0
10-2 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.250	.108	12	4
	13	48.000	Complete	.188	.098	13	3
	14	72.000	Complete	.125	.083	14	2
	15	96.000	Censored	.	.	14	1
	16	96.000	Censored	.	.	14	0
10-3 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.500	.125	8	8
	9	48.000	Complete	.438	.124	9	7
	10	72.000	Complete	.375	.121	10	6
	11	96.000	Censored	.	.	10	5
	12	96.000	Censored	.	.	10	4
	13	96.000	Censored	.	.	10	3
	14	96.000	Censored	.	.	10	2
	15	96.000	Censored	.	.	10	1
	16	96.000	Censored	.	.	10	0

Means and Medians for Survival Time

Group	Mean[a]				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Original O/N culture	24.000	.000	24.000	24.000	24.000	.	.	.
10-1 dilution	33.125	5.410	22.522	43.728	24.000	.	.	.
10-2 dilution	37.500	6.353	25.048	49.952	24.000	.	.	.
10-3 dilution	55.500	8.410	39.016	71.984	24.000	.	.	.
Overall	37.781	3.373	31.170	44.392	24.000	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	12.754	3	.005

Test of equality of survival distributions for the different levels of Group.

11.1.7 A2 Strain: Survival and mean tables

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Original O/N culture	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.250	.108	12	4
	13	48.000	Complete	.188	.098	13	3
	14	72.000	Complete	.125	.083	14	2
	15	96.000	Censored	.	.	14	1
	16	96.000	Censored	.	.	14	0
10-1 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.563	.124	7	9
	8	48.000	Complete	.	.	8	8
	9	48.000	Complete	.438	.124	9	7
	10	72.000	Complete	.	.	10	6
	11	72.000	Complete	.313	.116	11	5
	12	96.000	Complete	.250	.108	12	4
	13	96.000	Censored	.	.	12	3
	14	96.000	Censored	.	.	12	2
	15	96.000	Censored	.	.	12	1
	16	96.000	Censored	.	.	12	0
10-2 dilution	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.875	.083	2	14
	3	48.000	Complete	.	.	3	13
	4	48.000	Complete	.	.	4	12
	5	48.000	Complete	.	.	5	11
	6	48.000	Complete	.625	.121	6	10
	7	72.000	Complete	.563	.124	7	9
	8	96.000	Censored	.	.	7	8
	9	96.000	Censored	.	.	7	7
	10	96.000	Censored	.	.	7	6
	11	96.000	Censored	.	.	7	5
	12	96.000	Censored	.	.	7	4
	13	96.000	Censored	.	.	7	3
	14	96.000	Censored	.	.	7	2
	15	96.000	Censored	.	.	7	1
	16	96.000	Censored	.	.	7	0
10-3 dilution	1	48.000	Complete	.	.	1	15
	2	48.000	Complete	.	.	2	14
	3	48.000	Complete	.	.	3	13
	4	48.000	Complete	.750	.108	4	12
	5	72.000	Complete	.688	.116	5	11
	6	96.000	Complete	.	.	6	10
	7	96.000	Complete	.563	.124	7	9
	8	96.000	Censored	.	.	7	8
	9	96.000	Censored	.	.	7	7
	10	96.000	Censored	.	.	7	6
	11	96.000	Censored	.	.	7	5
	12	96.000	Censored	.	.	7	4
	13	96.000	Censored	.	.	7	3
	14	96.000	Censored	.	.	7	2
	15	96.000	Censored	.	.	7	1
	16	96.000	Censored	.	.	7	0

Means and Medians for Survival Time

Group	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Original O/N culture	37.500	6.353	25.048	49.952	24.000	.	.	.
10-1 dilution	55.500	8.206	39.415	71.585	48.000	23.812	1.329	94.671
10-2 dilution	73.500	6.864	60.047	86.953	.	.	.	.
10-3 dilution	82.500	5.598	71.528	93.472	.	.	.	.
Overall	62.250	4.009	54.392	70.108	48.000	12.000	24.480	71.520

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	17.483	3	.001

Test of equality of survival distributions for the different levels of Group.

11.1.8 Kill kinetics

	Time (h)					
	4	6	8	10	12	24
Repeat 1	1.56E+04	1.58E+06	7.62E+06	3.32E+09	5.30E+09	4.27E+04
Repeat 2	1.37E+04	1.63E+06	5.02E+06	1.93E+09	2.02E+10	4.78E+04
Repeat 3	1.40E+04	1900000	5.77E+06	1.91E+09	9.44E+09	7.55E+05
Average CFU/ml	1.44E+04	1.70E+06	6.14E+06	2.39E+09	1.16E+10	2.82E+05
SD	1.02E+03	1.72E+05	1.34E+06	8.08E+08	7.69E+09	4.10E+05
SE	5.90E+02	9.94E+04	7.73E+05	4.67E+08	4.44E+09	2.37E+05

Analysis of kill kinetic test groups over 24 hour period

11.2 SECTION 5

Clear Well	Rifampicin (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (µg/ml)	FIC	Final Conc. (µg/ml)	FIC		
A8	2	MIC of A	0	-	-	-
B7	1	0.5	0.015	0.06	0.56	I
B6	0.5	0.25	0.015	0.06	0.31	S
B5	0.25	0.13	0.015	0.06	0.19	S
B4	0.13	0.07	0.015	0.06	0.13	S
C3	0.06	0.03	0.03	0.125	0.16	S
D3	0.06	0.03	0.06	0.25	0.31	S
E2	0.03	0.02	0.125	0.5	0.52	I
F2	0.03	0.02	0.25	1	1.02	I
F1	0	-	0.25	MIC of B	-	-

Table 4.1 Interpretation table of the checkerboard assay combining colistin with rifampicin.

Clear Well	Tetracycline (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (µg/ml)	FIC	Final Conc. (µg/ml)	FIC		
A7	1	MIC of A	0	-	-	-
B7	1	1	0.015	0.06	1.06	I
C7	1	1	0.03	0.13	1.13	I
D6	0.5	0.5	0.06	0.25	0.75	I
E5	0.25	0.25	0.125	0.5	0.75	I
F4	0.125	0.125	0.25	1	1.125	I
F3	0.06	0.06	0.25	1	1.06	I
F2	0.03	0.03	0.25	1	1.03	I
F1	0	-	0.25	MIC of B	-	-

Table 4.2 Interpretation table of the checkerboard assay combining colistin with tetracycline.

Clear Well	Kanamycin (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (µg/ml)	FIC	Final Conc. (µg/ml)	FIC		
A9	16	MIC of A	0	-	-	-
B9	16	1	0.015	0.06	1.06	I
C9	16	1	0.03	0.12	1.12	I
D9	16	1	0.06	0.24	1.25	I
E8	8	0.5	0.125	0.5	1.00	I
E7	4	0.25	0.125	0.5	0.75	I
F6	2	0.125	0.25	1	1.13	I
F5	1	0.06	0.25	1	1.06	I
F4	0.5	0.03	0.25	1	1.03	I
F3	0.25	0.01	0.25	1	1.01	I
F2	0.125	0.005	0.25	1	1.005	I
F1	0	0	0.25	MIC of B	-	-

Table 4.3 Interpretation table of the checkerboard assay combining colistin with kanamycin

Clear Well	Erythromycin (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (μg/ml)	FIC	Final Conc. (μg/ml)	FIC		
A10	32	MIC of A	0	-	-	-
B9	16	0.5	0.015	0.06	0.56	I
C9	16	0.5	0.03	0.12	0.62	I
D8	8	0.25	0.06	0.2	0.49	S
D7	4	0.125	0.06	0.2	0.37	S
E6	2	0.06	0.125	0.5	0.56	I
E5	1	0.03	0.125	0.5	0.53	I
F4	0.5	0.01	0.25	1	1.01	I
F3	0.25	0.007	0.25	1	1.007	I
F2	0.125	0.003	0.25	1	1.003	I
F1	0	-	0.25	MIC of B	-	-

Table 4.4 Interpretation table of the checkerboard assay combining colistin with erythromycin.

Clear Well	Ciprofloxacin (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (μg/ml)	FIC	Final Conc. (μg/ml)	FIC		
A9	16	MIC of A	0	-	-	-
B9	16	1	0.015	0.06	1.06	I
D8	8	0.5	0.06	0.24	0.74	I
E7	4	0.25	0.125	0.5	0.75	I
E6	2	0.125	0.125	0.5	0.625	I
F5	1	0.06	0.25	1	1.06	I
F4	0.5	0.03	0.25	1	1.03	I
F3	0.25	0.01	0.25	1	1.01	I
F2	0.125	0.005	0.25	1	1.005	I
F1	0	-	0.25	MIC of B	-	-

Table 4.5 Interpretation table of the checkerboard assay combining colistin with ciprofloxacin.

Clear Well	Gentamicin (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (μg/ml)	FIC	Final Conc. (μg/ml)	FIC		
A10	32	MIC of A	0	-	-	-
B9	32	1	0.015	0.06	1.06	I
D8	16	0.5	0.06	0.24	0.74	I
E7	8	0.25	0.125	0.5	0.75	I
F5	2	0.063	0.25	1	1.06	I
F4	1	0.031	0.25	1	1.03	I
F3	0.5	0.016	0.25	1	1.02	I
F2	0.25	0.008	0.25	1	1.005	I
F1	0	-	0.25	MIC of B	-	-

Table 4.6 Interpretation table of the checkerboard assay combining colistin with gentamicin.

Clear Well	Chloramphenicol (A)		Colistin (B)		ΣFIC	Interpretation
	Final Conc. (μg/ml)	FIC	Final Conc. (μg/ml)	FIC		
A10	32	MIC of A	0	-	-	-
B9	32	1	0.015	0.06	1.06	I
D8	16	0.5	0.06	0.24	0.74	I
E7	8	0.25	0.125	0.5	0.75	I
E6	4	0.125	0.125	0.5	0.63	I
F5	2	0.063	0.25	1	1.06	I
F4	1	0.031	0.25	1	1.03	I
F3	0.5	0.016	0.25	1	1.02	I
F2	0.25	0.008	0.25	1	1.005	I
F1	0	-	0.25	MIC of B	-	-

Table 4.7 Interpretation table of the checkerboard assay combining colistin with chloramphenicol.

11.3 SECTION 6

	Repeat				
Time	1	2	3	Average	Standard Dev
24	31.25	31.25	25	29.16667	3.608439
48	31.25	18.75	18.75	22.91667	7.216878
72	12.5	12.5	12.5	12.5	0
96	18.75	12.5	12.5	14.58333	3.608439

Treatment of biofilm grown ATCC 19606 with colistin

	Repeat				
Time	1	2	3	Average	Standard Dev
24	31.25	31.25	25	29.16667	3.608439
48	12.5	18.75	18.75	16.66667	3.608439
72	12.5	6.25	6.25	8.333333	3.608439
96	0	6.25	6.25	4.166667	3.608439

Treatment of biofilm grown ATCC 19606 with erythromycin

	Repeat				
Time	1	2	3	Average	Standard Dev
24	31.5	25	18.75	25.08333	6.375408
48	12.5	25	18.75	18.75	6.25
72	6.25	25	12.5	14.58333	9.547033
96	6.25	18.75	12.5	12.5	6.25

Treatment of biofilm grown ATCC 19606 with rifampicin

	Repeat				
Time	1	2	3	Average	Standard Dev
24	62.5	68.75	62.5	55.20833	3.608439
48	50	43.75	43.75	41.66667	3.608439
72	37.5	43.75	31.25	36.45833	6.25
96	31.25	43.75	31.25	35.41667	7.216878

Treatment of biofilm grown ATCC 19606 with colistin and erythromycin combination

	Repeat				
Time	1	2	3	Average	Standard Dev
24	68.75	75	68.75	70.83333	3.608439
48	62.5	62.5	68.75	64.58333	3.608439
72	62.5	50	56.25	56.25	6.25
96	43.75	37.5	43.75	41.66667	3.608439

Treatment of biofilm grown ATCC 19606 with colistin and rifampicin combination

F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	12.5	41.66666667
Variance	39.0625	13.02083333
Observations	3	3
df	2	2
F	3	
P(F<=f) one-tail	0.25	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	12.5	41.66666667
Variance	39.0625	13.02083333
Observations	3	3
Pooled Variance	26.04166667	
Hypothesized Mean Difference	0	

df	4
t Stat	-7
P(T<=t) one-tail	0.001096065
t Critical one-tail	2.131846786
P(T<=t) two-tail	0.00219213
t Critical two-tail	2.776445105

Planktonic ATCC19606 f-Test and T-Test comparison of the significance of treatment with rifampicin

F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	4.166666667	33.33333333
Variance	13.02083333	13.02083333
Observations	3	3
df	2	2
F	1	
P(F<=f) one-tail	0.5	
F Critical one-tail	0.052631579	

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	4.166666667	33.33333333
Variance	13.02083333	13.02083333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-9.89949494	
P(T<=t) one-tail	0.000292205	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.000584411	
t Critical two-tail	2.776445105	

Planktonic ATCC19606 f-Test and T-Test comparison of the significance of treatment with erythromycin

11.4 SECTION 7

11.4.1 Treatment of biofilm grown *A. baumannii* ATCC 19606

	Repeat				
Time	1	2	3	Average	Standard Dev
24	68.75	62.5	68.75	66.66667	3.608439
48	50	43.75	50	47.91667	3.608439
72	43.75	37.5	43.75	41.66667	3.608439
96	37.5	25	37.5	33.33333	7.216878

Colistin

	Repeat				
Time	1	2	3	Average	Standard Dev
24	37.5	37.5	43.75	39.58333	3.608439
48	25	25	43.75	31.25	10.82532
72	25	25	37.5	29.16667	7.216878
96	18.75	25	37.5	27.08333	9.547033

Erythromycin

	Repeat				
Time	1	2	3	Average	Standard Dev
24	75	68.75	68.75	70.83333	3.608439
48	68.75	68.75	68.75	68.75	0
72	68.75	56.25	56.25	60.41667	7.216878
96	50	43.75	31.25	41.66667	9.547033

Rifampicin

	Repeat				
Time	1	2	3	Average	Standard Dev
24	68.75	75	50	64.58333	13.01041
48	68.75	62.5	50	60.41667	9.547033
72	50	56.25	43.75	50	6.25
96	50	56.25	37.5	47.91667	9.547033

Colistin and Erythromycin

F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	33.33333333	72.91666667
Variance	52.08333333	13.02083333
Observations	3	3
df	2	2
F	4	
P(F<=f) one-tail	0.2	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	33.33333333	72.91666667
Variance	52.08333333	13.02083333
Observations	3	3
Pooled Variance	32.55208333	
Hypothesized Mean Difference	0	
df	4	
t Stat	-8.49705831	
P(T<=t) one-tail	0.000525985	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.00105197	
t Critical two-tail	2.776445105	

Biofilm ATCC 19606 f-Test and T-Test comparison of the significance of treatment with rifampicin

F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	47.91666667	33.33333333
Variance	91.14583333	52.08333333
Observations	3	3
df	2	2
F	1.75	
P(F<=f) one-tail	0.363636364	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	47.91666667	33.33333333
Variance	91.14583333	52.08333333
Observations	3	3
Pooled Variance	71.61458333	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.110579412	
P(T<=t) one-tail	0.051211767	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.102423533	
t Critical two-tail	2.776445105	

Biofilm ATCC 19606 f-Test and T-Test comparison of the significance of treatment with erythromycin

11.4.2 Planktonic and biofilm grown E.coli treatment

Case Processing Summary

Group	Total N	N of Events	Censored	
			N	Percent
Suture Control	16	0	16	100.0%
PBS control	16	0	16	100.0%
Plantonic E.coli Control	16	16	0	0.0%
Planktonic E.coli + Colistin	16	2	14	87.5%
Planktonic E. coli + Rifampicin	16	15	1	6.3%
Planktonic E. coli + Erythromycin	16	16	0	0.0%
Planktonic E. coli + Combination Therapy	16	0	16	100.0%
Biofilm E.coli Control	16	16	0	0.0%
Biofilm E.coli + Colistin	16	1	15	93.8%
Overall	144	66	78	54.2%

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Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Suture Control	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
PBS control	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
Plantonic E.coli Control	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.063	.061	15	1
	16	48.000	Complete	.000	.000	16	0
Planktonic E.coli + Colistin	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.875	.083	2	14
	3	96.000	Censored	.	.	2	13
	4	96.000	Censored	.	.	2	12
	5	96.000	Censored	.	.	2	11
	6	96.000	Censored	.	.	2	10
	7	96.000	Censored	.	.	2	9
	8	96.000	Censored	.	.	2	8
	9	96.000	Censored	.	.	2	7
	10	96.000	Censored	.	.	2	6
	11	96.000	Censored	.	.	2	5
	12	96.000	Censored	.	.	2	4
	13	96.000	Censored	.	.	2	3
	14	96.000	Censored	.	.	2	2
	15	96.000	Censored	.	.	2	1
	16	96.000	Censored	.	.	2	0
Planktonic E. coli + Rifampicin	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.125	.083	14	2
	15	72.000	Complete	.063	.061	15	1
	16	96.000	Censored	.	.	15	0
Planktonic E. coli + Erythromycin	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.063	.061	15	1
	16	48.000	Complete	.000	.000	16	0
Planktonic E. coli + Combination Therapy	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
Biofilm E.coli Control	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
Biofilm E.coli + Colistin	1	48.000	Complete	.938	.061	1	15
	2	96.000	Censored	.	.	1	14
	3	96.000	Censored	.	.	1	13
	4	96.000	Censored	.	.	1	12
	5	96.000	Censored	.	.	1	11
	6	96.000	Censored	.	.	1	10
	7	96.000	Censored	.	.	1	9
	8	96.000	Censored	.	.	1	8
	9	96.000	Censored	.	.	1	7
	10	96.000	Censored	.	.	1	6
	11	96.000	Censored	.	.	1	5
	12	96.000	Censored	.	.	1	4
	13	96.000	Censored	.	.	1	3
	14	96.000	Censored	.	.	1	2
	15	96.000	Censored	.	.	1	1
	16	96.000	Censored	.	.	1	0

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	140.271	8	.000

Test of equality of survival distributions for the different levels of Group.

11.4.3 Mixed biofilm treatment

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
Suture Control	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1
Biofilm E.coli Control	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
Biofilm A. baumannii Control	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.500	.125	8	8
	9	48.000	Complete	.	.	9	7
	10	48.000	Complete	.375	.121	10	6
	11	72.000	Complete	.	.	11	5
	12	72.000	Complete	.250	.108	12	4
	13	96.000	Complete	.188	.098	13	3
	14	96.000	Censored	.	.	13	2
	15	96.000	Censored	.	.	13	1
	16	96.000	Censored	.	.	13	0
Mixed biofilm control	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.	.	2	14
	3	24.000	Complete	.	.	3	13
	4	24.000	Complete	.	.	4	12
	5	24.000	Complete	.	.	5	11
	6	24.000	Complete	.	.	6	10
	7	24.000	Complete	.	.	7	9
	8	24.000	Complete	.	.	8	8
	9	24.000	Complete	.	.	9	7
	10	24.000	Complete	.	.	10	6
	11	24.000	Complete	.	.	11	5
	12	24.000	Complete	.	.	12	4
	13	24.000	Complete	.	.	13	3
	14	24.000	Complete	.	.	14	2
	15	24.000	Complete	.	.	15	1
	16	24.000	Complete	.000	.000	16	0
Mixed biofilm + Colistin	1	24.000	Complete	.	.	1	15
	2	24.000	Complete	.875	.083	2	14
	3	96.000	Complete	.813	.098	3	13
	4	96.000	Censored	.	.	3	12
	5	96.000	Censored	.	.	3	11
	6	96.000	Censored	.	.	3	10
	7	96.000	Censored	.	.	3	9
	8	96.000	Censored	.	.	3	8
	9	96.000	Censored	.	.	3	7
	10	96.000	Censored	.	.	3	6
	11	96.000	Censored	.	.	3	5
	12	96.000	Censored	.	.	3	4
	13	96.000	Censored	.	.	3	3
	14	96.000	Censored	.	.	3	2
	15	96.000	Censored	.	.	3	1
	16	96.000	Censored	.	.	3	0
Uninfected	1	96.000	Censored	.	.	0	15
	2	96.000	Censored	.	.	0	14
	3	96.000	Censored	.	.	0	13
	4	96.000	Censored	.	.	0	12
	5	96.000	Censored	.	.	0	11
	6	96.000	Censored	.	.	0	10
	7	96.000	Censored	.	.	0	9
	8	96.000	Censored	.	.	0	8
	9	96.000	Censored	.	.	0	7
	10	96.000	Censored	.	.	0	6
	11	96.000	Censored	.	.	0	5
	12	96.000	Censored	.	.	0	4
	13	96.000	Censored	.	.	0	3
	14	96.000	Censored	.	.	0	2
	15	96.000	Censored	.	.	0	1

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	89.180	5	.000

Test of equality of survival distributions for the different levels of Group.

11.4.4 Total Viable counts

N°	Count	Dilution	CFU/mL	CFU Prorata	Area (%)	mean CFU Ø (mm)	min CFU Ø (mm)	max CFU Ø (mm)	Date
1	237	1.00E-03	2.87E+06	287	83	0.95	0.63	1.56	05/02/2014
2	381	1.00E-02	4.06E+05	406	94	1.06	0.55	2.09	05/02/2014
3	79	1.00E-03	8.41E+05	84	94	1.01	0.63	1.65	05/02/2014
		Average	1.37E+06						

Raw data for total viable counts for *A. baumannii* ATCC 19606 cultured in a single species biofilm on to surgical suture.

N°	Count	Dilution	CFU/mL	CFU Prorata	Area (%)	mean CFU Ø (mm)	min CFU Ø (mm)	max CFU Ø (mm)	Date
A.baumannii 1	176	1.00E-07	4.84E+08	3	3.63E+08	0	0	5	12/02/2014
A.baumannii 2	294	1.00E-06	3.39E+08	26	3.15E+08	2	24200000	34	12/02/2014
A.baumannii 3	235	1.00E-05	2.85E+08	228	2.76E+08	0	0	285	12/02/2014
		Average	3.69E+08						

Raw data for total viable counts for *A. baumannii* ATCC 19606 cultured in a mixed species biofilm on to surgical suture.

Sample N°	Count	Dilution	CFU/mL	CFU Prorata	Area (%)	mean CFU Ø (mm)	min CFU Ø (mm)	max CFU Ø (mm)	Date
E.coli G 2	227	1.00E-06	2.72E+09	272	83	2.43	0.78	5.75	21/03/2014
E.coli G 3	43	1.00E-07	5.85E+09	59	73	3.69	1.09	6.87	21/03/2014
E.coli G 4	8	1.00E-08	1.16E+10	12	69	3.98	3.15	6.23	21/03/2014
		Average	4.29E+09						

Raw data for total viable counts for *E. coli* ATCC 25922 cultured in a mixed species biofilm on to surgical suture.

Sample N°	Count	Dilution	CFU/mL	Class 1	CFU Prorata	Area (%)	mean CFU Ø (mm)	min CFU Ø (mm)	max CFU Ø (mm)	Date
E.coli 1	9	1.00E-07	9.17E+08	9	9	98	3.6	0.63	6.25	23/04/2014
E.coli 2	84	1.00E-06	9.44E+08	84	94	89	2.03	0.59	4.4	23/04/2014
E.coli 3	199	1.00E-05	2.24E+08	197	224	89	1.71	0.59	4.07	23/04/2014
		Average	6.95E+08							

Raw data for total viable counts for *E. coli* ATCC 25922 cultured in a single species biofilm on to surgical suture.